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Evidence for a Positive Role of Transforming Growth Factor-β in Human Breast Cancer Cell Tumorigenesis

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Abstract. To determine the biological role of transforming growth factor-β (TGF-β) in mammary carcinomas *in vivo*, estrogen-dependent MCF-7 cells were transfected with a mouse TGF-β1 cDNA. Growth characteristics in culture were not altered in the transfected cells. However, the MCF-7/TGF-β1 cells formed tumors in ovariectomized athymic mice in the absence of estrogen supplementation. Daily injections of human recombinant TGF-β1 supported tumor formation by wild-type MCF-7 cells in castrated nude mice in the absence of exogenous estradiol. In another approach to the same question, the effect of anti-TGF-β antibodies on tumor formation by estrogen-independent MDA-231 cells was examined. The 2G7 IgG2b (2G7) antibody, which neutralizes TGF-β1, -β2, and -β3, blocked the formation of MDA-231 tumors at the injection site and lung metastases in nude mice. Inoculation of MDA-231 cells inhibited, while injection of 2G7 increased mouse spleen natural killer (NK) activity. 2G7 did not inhibit MDA-231 tumors and metastases in NK-deficient animals. Finally, medium conditioned by MDA-231 cells inhibited lymphocyte-mediated NK activity; this inhibition was abrogated by 2G7, but not by a control IgG2. These data support a positive role for tumor cell TGF-β in the maintenance and/or progression of mammary carcinoma cells in an intact host.

Key words: Breast neoplasms, estrogen-dependent growth, immunologic surveillance, natural killer activity, nude mice, transforming growth factor-β

Transforming growth factor (TGF)-β1, -β2, and -β3 are expressed by human breast cancer cells in culture [1,2]. Exogenous TGF-β1 and -β2 inhibit the proliferation of most breast cancer cells *in vitro* [3–5], but fail to inhibit the growth of (*in vitro*-sensitive) mammary tumors in nude mice [6]. On the other hand, higher levels of TGF-β1 expression are circumstantially associated with a more aggressive breast tumor phenotype in either cultured cell lines or human mammary tissues [7–13]. In an effort to understand the biological role of tumor cell TGF-β expression in human mammary cancer, we have manipulated the TGF-β system in human breast cancer cell lines and examined the effect of these manipulations on the tumorigenic potential of these cells in athymic mice.

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TGF-β1 CAN INDUCE ESTROGEN-INDEPENDENT TUMORIGENICITY OF HUMAN BREAST CANCER CELLS IN NUDE MICE

The MCF-7 breast cancer cell line was transfected with expression vectors pSVTGF-β1 and pZip*neo* (which confers neomycin resistance) or with pZip*neo* alone [14]. The pSVTGF-β1 vector contains a full-length mouse TGF-β1 cDNA encoding the latent TGF-β1 protein. After selection in G418, an MCF-7/TGF-β1 clone was isolated that secreted >10-fold more TGF-β activity than wild-type MCF-7 or MCF-7/*neo* cells as measured in a ^{125}I-TGF-β2 radioreceptor assay [14]. The effect of TGF-β1 overexpression on the ability of the MCF-7 cells to form tumors in ovariectomized nude mice was examined. Mice were inoculated subcutaneously (sc) with 5×10^6 cells. In some cases a 60-day release, 0.7 mg 17β-estradiol pellet was implanted (sc) distant from the tumor site one day prior to tumor cell inoculation. In some animals, serial intraperitoneal (ip) injections of 200 μg of the anti-TGF-β monoclonal antibodies 12H5 or 2G7 were given the day of tumor cell inoculation and continued every 3 days for 2–3 weeks. 2G7 is an IgG2b monoclonal antibody that neutralizes TGF-β1, -β2, and -β3, whereas 12H5 is a non-neutralizing (control) IgG2a [15]. Results are shown in Table I.

The TGF-β1-transfected MCF-7 cells formed tumors in the absence of exogenous estradiol approximately half the size of estrogen-primed wild-type tumors. Both tumor types were histologically indistinguishable [14]. Tumor formation by the transfected cells was abrogated by 2G7, but not by the 12H5 control antibody. 17β-estradiol stimulated growth of the MCF-7/TGF-β1 tumors. MCF-7/*neo* cells did not form tumors in mice in the absence of estrogen supplementation. Contrary to the MCF-7/TGF-β1 tumors, estrogen-primed wild-type MCF-7 tumors were not inhibited by 2G7, supporting the notion that 2G7-mediated inhibition of tumors overexpressing TGF-β1 was due to specific neutralization of the transfected gene.

TABLE I

Cell line	E2	Antibody	Tumors formed	Tumor size (mm^3)[c]
MCF-7	+	none	6/6	448 ± 114
MCF-7	–	none	0/4	
MCF-7/ TGF-β1	–	12H5[a]	6/6	158 ± 37
MCF-7/ TGF-β1	–	2G7[a]	0/6	
MCF-7/ TGF-β1	+	none	6/6	490 ± 152
MCF-7/*neo*	–	none	0/6	
MCF-7	+	12H5[b]	3/3	154 ± 42
MCF-7	+	2G7[b]	3/3	178 ± 60

Ovariectomized nude mice were inoculated sc with 5×10^6 tumor cells. In some cases, animals were supplemented with slow-release 17β-estradiol (E2) pellets. Monoclonal antibodies 12H5 or 2G7 were given ip every 3 days for the first 2[b] or 3[a] weeks. Data shown represent the means ± SE of 3–6 mice per group. The first six and the last two experimental groups were done simultaneously. For the first group, 5-week tumor incidence and volumes are reported, whereas for the second group, 3-week tumor data are included.

[c] calculated by formula: $\text{width}^2 \times \text{length}/2$

EXOGENOUS TGF-β1 CAN SUPPORT ESTROGEN-INDEPENDENT TUMOR FORMATION BY WILD-TYPE MCF-7 CELLS IN NUDE MICE

Several of the characteristics exhibited by MCF-7 cells in culture were not altered by the TGF-β1 transfection. These included proliferation in monolayer, colony formation in soft agarose, sensitivity to exogenous TGF-β1, TGF-β binding, content of type I and III affinity cross-linked TGF-β receptors, sensitivity to estradiol or the antiestrogen tamoxifen, EGF binding sites, and cell content and secretion of TGF-α [14]. We therefore speculated that the TGF-β1-mediated enhanced tumorigenesis was probably not due to a direct effect of the transfected gene on the MCF-7 cells themselves, but rather to an effect of the transgenic protein product on the host which, in turn, favored the establishment and progression of the transfected cells as xenografts. Consistent with this speculation, daily ip injections of 1 μg human recombinant TGF-β1 transiently supported the growth of wild-type MCF-7 tumors in castrated nude mice in the absence of estradiol supplementation (Fig. 1).

NEUTRALIZATION OF TGF-βs WITH 2G7 ABROGATES MDA-231 LOCAL TUMOR AND LUNG METASTASES FORMATION IN NK-COMPETENT BUT NOT IN NK-DEFICIENT NUDE MICE

Once we determined that TGF-β1 overexpression in estrogen-responsive MCF-7 cells can contribute to their escape from hormone dependence, the next question was whether antibody-mediated blockade of TGF-β would inhibit breast tumor formation in an intact host. For these studies, we used the human breast cancer cell line MDA-231, which expresses high levels of TGF-β1 and -β2. When injected ip, these cells

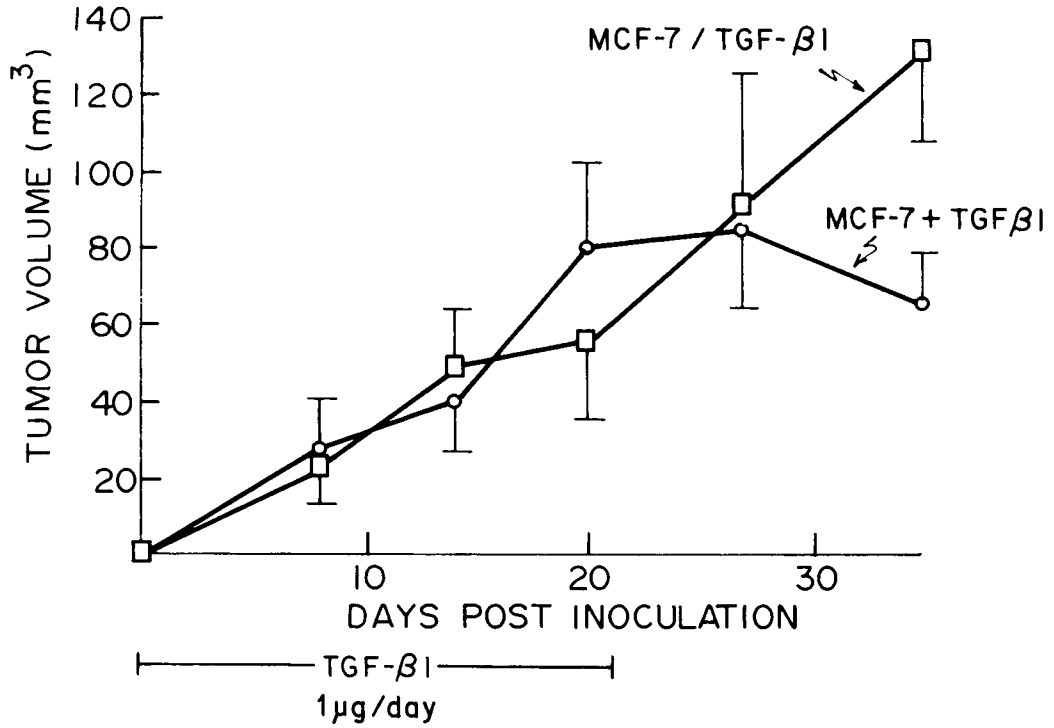

Fig 1. Effect of exogenous TGF-β1 on wild-type MCF-7 tumor formation in estrogen-free athymic mice. Ovariectomized nude mice were inoculated sc in the flank just caudal to the forelimb with $5x10^6$ wild-type MCF-7 (○) or MCF-7/TGF-β1 cells (□). Estradiol pellets were not administered. Mice inoculated with wild-type cells received 1 μg human recombinant TGF-β1 ip daily for a total of 3 weeks. Each data point represents the mean ± SE of 6 mice. Reproduced with permission from Cell Growth & Differentiation.

rapidly form extensive intra-abdominal tumors and hematogenous lung metastases [16]. NK-competent (NK+) female athymic mice were inoculated ip with 5×10^6 MDA-231 cells, followed 24 hours later by every-other-day ip injections of phosphate-buffered saline (PBS) or 200 μg of the 12H5 or 2G7 monoclonal antibodies. After three weeks, mice were sacrificed and multiple specimens from different sites in the abdominal cavity and lungs collected and assessed for the presence of tumor cells by light microscopy. In some cases, mouse spleens were aseptically removed and a single mouse spleen cell suspension prepared. ^{51}Cr-labeled YAC-1 mouse lymphoma target cells were incubated with the effector mouse spleen cells, and the amount of radioisotope released was measured to calculate mouse spleen NK activity as described [16].

Neutralization of TGF-β with 2G7 suppressed the development of MDA-231 intra-abdominal tumors and detectable lung metastases in NK+ mice (Table II). All animals treated with the 12H5 non-neutralizing control IgG2a and PBS exhibited extensive omental seeding by tumor cells and metastases in both lungs at three weeks. The anti-tumor effect of 2G7 was dose dependent; it was less marked with 20 μg injections and almost absent with 2 μg injections (not shown, but detailed in Ref. 16).

We then examined the effect of 2G7 on mouse immune function, which in turn may explain the potent anti-tumor effect. Mouse spleen NK function measured as ^{51}Cr release from YAC-1 syngeneic lymphoma cells, was high in the 2G7-treated animals but low in those treated with PBS and 12H5 (Table II). To test whether NK activity was critical for the anti-tumor effect, a similar experiment was performed in beige NK-deficient nude mice. In contrast to the previous result, all mice treated with 12H5 or 2G7 developed extensive ip MDA-231 tumors and large metastatic foci in both lungs. This result suggests that, in an intact host, the endogenously produced TGF-β can regulate immune function in an autocrine fashion, and that upregulation of this immune parameter is critical for the observed anti-tumor effect. It is also consistent with the reported inhibitory effect of TGF-β on natural killer cells [17].

MDA-231 CELLS SECRETE TGF-β ACTIVITY THAT INHIBITS LYMPHOCYTE-MEDIATED NK ACTIVITY

Simultaneously with the experiments summarized in Table II and confirming a previous report [18], we observed that the inoculation of MDA-231 tumor cells would inhibit basal spleen NK function in athymic mice [16], suggesting the possibility that this immune downregulation was

TABLE II. Effect of TGF-β Blockade on MDA-231 Tumor and Metastases Formation

Treatment	Mouse NK status	Ip tumors	Lung metastases	Spleen NK activity
PBS	NK+	6/6	6/6	Low
12H5	NK+	6/6	6/6	Low
2G7	NK+	0/6	0/6	High
12H5	NK–	6/6	6/6	UD
2G7	NK–	6/6	6/6	UD

NK+ or NK– female nude mice were inoculated ip with 5×10^6 MDA-231 cells. The following day, every-other-day ip injections of PBS, 12H5, or 2G7 were started. After 8–10 doses (3 weeks), mice were sacrificed and the abdominal cavity and lungs examined macroscopically and microscopically for the presence of tumor cells. In some cases, spleens were harvested and the NK activity of a spleen cell suspension measured as described previously [16].

UD = undetectable

important for tumor progression. To follow this observation, and because of our inability to establish the relative contribution of NK down-regulation to MDA-231 cell tumorigenesis, which would require the co-administration of exogenous NK cells and tumor cells to nude mice, we tested the effect of MDA-231-cell conditioned medium (CM) on human lymphocyte NK function. NK activity was measured as isotope release from ^{51}Cr-labeled human K-562 erythroleukemia cells incubated with human lymphocytes previously treated with CM. Compared to unconditioned (control) medium, CM from MDA-231 cells inhibited lymphocyte NK function. This inhibition was partially blocked by 2G7, but not by a control IgG2 (Fig. 2), supporting the notion that tumor cell TGF-β may contribute to the tumorigenic process by suppressing mechanisms of immune surveillance in the host.

BIOLOGICAL IMPLICATIONS AND FUTURE STUDIES

The data presented here support a causal role for tumor cell TGF-β in the development of hormonal autonomy and/or progression of human breast carcinomas. These data are also consistent with the reported association of higher levels of TGF-β expression with the development of estrogen independence by breast cancer lines in culture [7,8], the absence of hormone receptors in human breast tumors [10], the progression from *in situ* to invasive breast carcinomas [11], the development of tamoxifen resistance by mamma-

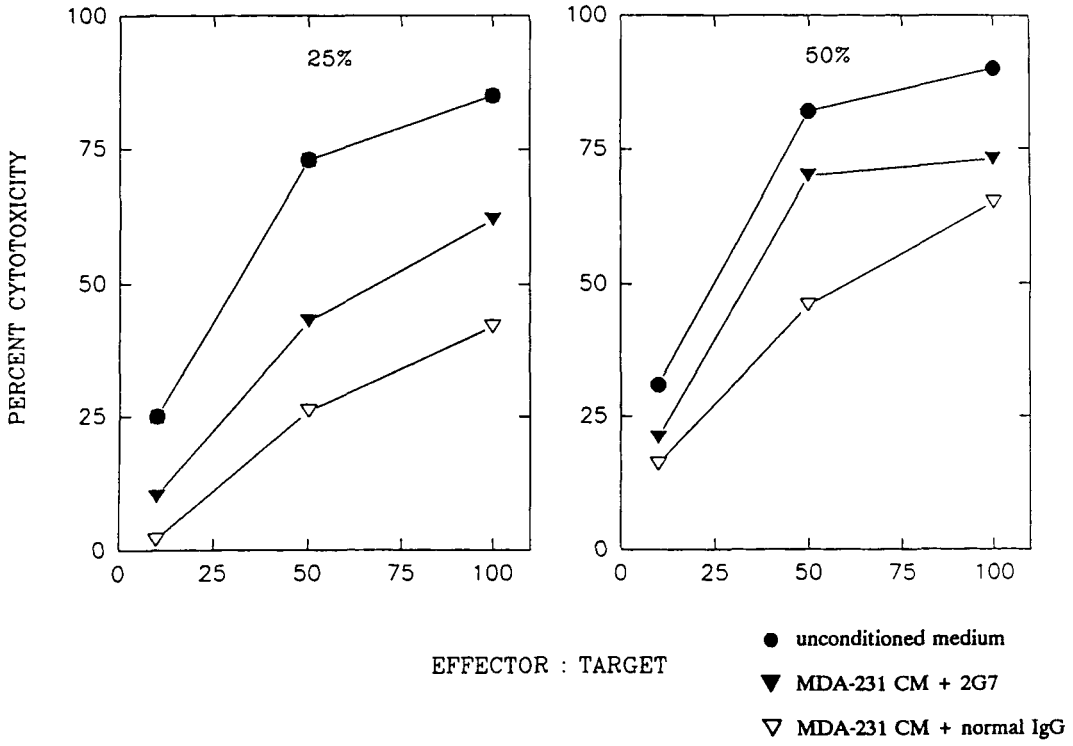

Fig. 2. Effect of anti-TGF-β neutralizing antibodies on MDA-231-cell CM-mediated inhibition of lymphocyte NK function. Serum-free CM from MDA-231 cells was preincubated for 6 hours at 4°C with 10 μg/ml 2G7 or a nonspecific IgG2 (Sigma). Control (unconditioned) medium or CM, in concentrations of 25% and 50%, were then added to peripheral blood lymphocytes for 18 hours. After washes, lymphocytes were added to ^{51}Cr-labeled K-562 cells for 4 hours at 37°C and lymphocyte NK function measured as radioisotope release from target cells as described previously [16]. Reproduced from the Journal of Clinical Investigation, (due out December, 1993) by copyright permission of the American Society for Clinical Investigation.

ry tumors [12], and a shorter disease-free interval after mastectomy [13]. On the other hand, these data are not consistent with the reported induction of TGF-β1 protein in breast cancer cell lines [3] and in mammary tissues [19] in response to the antiestrogen tamoxifen. However, experimental evidence that TGF-β induction is required for tamoxifen-mediated growth inhibition of breast cancer cells is still lacking.

Similar observations have been made in other experimental systems. For example, highly immunogenic UV-induced fibrosarcoma cells transfected with a mouse TGF-β1 cDNA exhibited enhanced tumorigenicity in nude mice and, contrary to the parental cells, were unable to induce cytotoxic mouse lymphocyte responses [20]. CHO cells, transfected with a TGF-β1 expression vector encoding latent TGF-β1, decreased NK cell activity and rapidly formed tumors in athymic mice [21]. MATLyLu rat prostate cancer cells overexpressing pSVTGF-β1 produced more metastatic tumors than untransfected cells [22]. TGF--β1-transfected human E1A-transformed 293 tumor cells were more tumorigenic than wild-type cells in athymic mice, and displayed increased adhesiveness *in vitro* [23]. Finally, Meth A sarcoma cells, transfected with a TGF-β1 expression plasmid encoding an activated TGF-β1 protein, display profound growth inhibition and increased adhesiveness *in vitro*. However, these cells were much more tumorigenic than the parental cells in immunocompetent mice, inducing highly anaplastic tumors and marked splenomegaly in the host [24]. This particular result underscores the potential flaw of extrapolating *in vitro* effects displayed by a multipotent molecule like TGF-β to those displayed in an intact host.

In contrast to studies with human tumor cells *in vivo*, several studies have shown that TGF-β1 inhibits normal mammary gland development in the mouse [25,26]. Moreover, transgenic mice overexpressing TGF-β1 exhibit mammary alveolar atrophy and fail to lactate [27]. These data do not necessarily contradict the data on human tumor cells summarized above. They suggest, though, that TGF-β may have a different role depending on the phenotype and/or microenvironment of the mammary epithelial cell. Another possibility is that TGF-β's role changes at some point during the progression to preneoplasia and subsequent *in situ* and invasive carcinomas. Future studies examining the expression of different TGF-β isoforms and receptors in progressively transformed human mammary lesions will hopefully shed light into these speculations.

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