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Importance of Transforming Growth Factor α/Epidermal Growth Factor Receptor Autocrine Growth Mechanism in an Ovarian Cancer Cell Line *in Vivo*

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ABSTRACT

We have elucidated the importance of a transforming growth factor (TGF) α and epidermal growth factor receptor autocrine mechanism on the growth of a human ovarian serous cystadenocarcinoma-derived cell line (SHIN-3) *in vitro*. In this study, we studied the biological significance of this autocrine mechanism *in vivo* using female athymic nude (*nu/nu*) mice. We measured the mouse plasma epidermal growth factor and TGFα levels by radioimmunoassay and enzyme-linked immunosorbent assay, respectively. Plasma epidermal growth factor concentrations were remarkably decreased by sialoadenectomy (Sx): 410 ± 65 (SE) pg/ml (n = 10) in intact animals; and undetectable in Sx mice (n = 5). Plasma TGFα levels were 90 and 40 pg/ml in intact and in Sx animals, respectively. Ten million SHIN-3 cells inoculated into *nu/nu* mice formed tumors in 100% of mice, and tumors grew progressively. Implantabilities and tumor growth rates of inoculated cells were not affected by Sx and even by Sx and anti-mouse epidermal growth factor antibody treatment. However, anti-TGFα monoclonal antibody (mAb) administered to SHIN-3 cell-inoculated Sx animals drastically reduced the tumor growth. Although 10^7 SHIN-3 cells formed tumors in this group, tumor growth was significantly inhibited by 10 μg of anti-TGFα mAb given 3 times a week, and growth inhibitions were more by 20 μg of anti-TGFα mAb. Moreover, as aggressive tumor growth as that in Sx animals was resumed by the cessation of anti-TGFα mAb treatments. All these data suggested the biological importance of a TGFα/epidermal growth factor receptor autocrine mechanism on the growth of this cell line *in vivo*.

INTRODUCTION

It is known that EGF[2] is mainly produced in the SMG, the main source of circulating EGF in mice (1). Sx in mice results in the elimination of plasma EGF (1). Sx causes various lesions in mice including decreased milk production in lactating animals (2), oligozoospermia (3), abortion in pregnant mice (4), and abnormal skin (5). Anti-mouse EGF antiserum administration to Sx animals causes more profound lesions than Sx alone (4, 5), and EGF replacement restores these lesions (2–5).

It is known that EGF-dependent mouse mammary tumor (6) inoculated into *nu/nu* mice is less efficiently transplanted and grown in Sx mice than in intact animals, and anti-EGF antiserum administration to *nu/nu* mice abolishes the implantation of the tumor (7). Moreover, EGF administration to Sx *nu/nu* mice restores the implantability and tumor growth of the mouse mammary tumor at rates higher than those in controls (7). This *nu/nu* mouse model would be available to human tumors in which EGF plays a role in their tumorigenesis and/or tumor growth because mouse EGF can affect human cells (8). This model also is useful in examining the role of a TGFα/EGFR growth mechanism *in vivo* because EGF and TGFα share a common receptor (9) and both of these growth factors have similar biological activities (10).

Growth mechanisms of ovarian cancers have not been well known. Elevated levels of TGFα are reported in the urine (11) and in the ascites (12) from patients with disseminated ovarian cancers and EGFR is commonly expressed in human ovarian cancer tissues (13). These data suggest the possible presence of a TGFα/EGFR autocrine mechanism in these tumors. Recently we have elucidated the expression and the biological significance of the TGFα/EGFR autocrine growth mechanism in a human ovarian serous cystadenocarcinoma-derived cell line [SHIN-3 (14)] *in vitro*.[3] In this study, we examined the role of a TGFα/EGFR autocrine growth mechanism of SHIN-3 cells *in vivo* using Sx *nu/nu* mice.

MATERIALS AND METHODS

Materials and Cells. Materials were purchased as follows: $Na^{125}I$ from New England Nuclear (Boston, MA); mouse EGF and anti-mouse EGF rabbit antiserum from Collaborative Research, Inc. (Bedford, MA); anti-TGFα mAb from Oncogene Science, Inc. (Manhasset, NY). Materials containing preservatives (sodium azide and others) were dialyzed against 0.01 M PBS, pH 7.4, at 4°C for 48 h and used *in vivo*. An ELISA kit for TGFα assay was kindly supplied by Dr. Shigeaki Tanaka, Hoechst Japan, Ltd. (Saitama, Japan).

SHIN-3 cells derived from human ovarian serous cystadenocarcinoma (14) were grown to subconfluence in a 75-cm^2 tissue culture flask (Corning Glass Works, Corning, NY) in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Cell Culture Laboratories, Cleveland, OH) at 37°C in a humidified 95% air-5% CO_2 atmosphere. Cells were trypsinized and washed with the serum-free RPMI 1640 by centrifugation. Ten million SHIN-3 cells in 0.2 ml of RPMI 1640 were inoculated into *nu/nu* mice s.c. according to the method of Satyaswaroop *et al.* (15).

Animals. Female BALB/c *nu/nu* mice, 6 weeks old, were purchased from SLC (Shizuoka, Japan) and maintained in our animal facilities as described previously (16). Some mice received Sx and others were sham-operated at 8 weeks of age. Two weeks after the operation, they were divided into 7 groups and 10^7 SHIN-3 cells were inoculated into each animal: group 1, sham-operated and no further treatments (controls, n = 3); group 2, Sx and no further treatments (n = 2); group 3, Sx and 100 μl of nonimmune rabbit serum (Bethyl Laboratories, Inc., Montgomery, TX) injected daily (n = 2); group 4, Sx and 100 μl of anti-mouse EGF rabbit antiserum given daily (n = 3); group 5, Sx and 20 μg of nonimmune mouse IgG (Zymed, San Francisco, CA) in 100 μl of PBS injected 3 times a week (n = 2); group 6, Sx and 10 μg of anti-TGFα mAb in 100 μl of PBS administered 3 times a week (n = 3); group 7, Sx and 20 μg of anti-TGFα mAb in 100 μl of PBS administered 3 times a week (n = 3). All treatments were begun at the time of tumor cell inoculation and were continued for 4 weeks. Tumors were measured in length and width twice a week throughout the study period and tumor sizes were expressed in (length) × $(\text{width})^2/2$.

RIA for EGF and ELISA for TGFα. EGF levels in mouse SMG and plasma were measured by the liquid phase double antibody method using ^{125}I-EGF as described previously (1). Mouse plasma samples were obtained from intact (n = 10) and Sx (n = 5) animals by bleeding into

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[2] The abbreviations used are: EGF, epidermal growth factor; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; *nu/nu* mice, athymic nude mice; SMG, submandibular gland; Sx, sialoadenectomy (surgical removal of SMG); RIA, radioimmunoassay; PBS, phosphate-buffered saline; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

[3] Submitted for publication.

heparinized syringes from the trunk vein under ether anesthesia. Blood samples were soon chilled on ice and centrifuged, and plasma fractions were stored at −20°C until assay. Submandibular gland tissues excised from mice were weighed and homogenized using a Polytron after the addition of 20 ml ice-cold PBS/g wet tissue. The homogenates were centrifuged at 12,000 × *g* for 20 min at 4°C and the supernatants were removed and stored at −20°C until assay.

Plasma TGFα levels were measured using the ELISA kit as described previously (17). Briefly, 3 ml of mouse plasma samples obtained from intact and Sx animals (3–4 animals in each group) were acidified, applied to Sep-Pak C_{18} cartridges (Millipore Co., Milford, MA), and TGFα was eluated. The eluates were lyophilized and dissolved in 0.01 M PBS, pH 7.2, and TGFα was measured by a sandwich-type ELISA.

Analysis of Data of Tumor Growth. Data are shown as the mean ± SE. Homoscedasticity of data was analyzed by the Bartlett test. The significance of differences was assessed by analysis of variance, followed by multiple comparisons of Dunett or Tukey, and $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Two million SHIN-3 cells inoculated into intact *nu/nu* mice formed tumors in 50% and more than 5 million cells grew tumors in 100% (data not shown). These transplantability rates seemed reasonable in comparison with the previous report (18). In this study, we inoculated 10^7 cells/mouse which developed a rapidly growing tumor and killed the mouse in 6–7 weeks.

We have elucidated that a TGFα/EGFR autocrine mechanism played an important role on the growth of this cell-line *in vitro*.[3] Therefore, EGF and/or TGFα in the mouse circulation might have growth-promoting effects on the inoculated SHIN-3 cells. We measured EGF concentrations in the SMG and the plasma in intact and Sx *nu/nu* mice by RIA. EGF contents in the SMG were 36.5 ± 3.5 ng/mg wet tissue ($n = 10$), at 8 weeks of age. Plasma EGF levels were 410 ± 65 pg/ml ($n = 10$) in controls and they were undetectable in Sx mice ($n = 5$, the sensitivity of our RIA being 50 pg/ml). These results were in good agreement with the previous report (7). We also measured plasma TGFα levels in intact and Sx *nu/nu* mice. Plasma TGFα concentrations were 90 and 40 pg/ml in intact and Sx animals, respectively. Although the values of plasma EGF and TGFα concentrations were not comparable simply because of the difference in the assay systems, the demonstration that plasma TGFα levels were lower than those of EGF seemed reasonable because TGFα production is limited in adult animals (19). The reason why circulating TGFα levels were lower in Sx animals than those in intact mice was not well known. However, our data showing that plasma EGF and TGFα were very low in Sx animals and the previous report that EGF-dependent mouse mammary tumor was less efficiently implanted and grown in Sx *nu/nu* mice and that it was not implantable to Sx and anti-EGF-treated animals suggested that little and no biologically significant amounts of growth factors to EGFR were available in Sx and in Sx and anti-EGF-treated *nu/nu* mice, respectively.

In this study, we found that 10^7 SHIN-3 cells were implantable and developed tumors in 100% of both Sx and Sx and anti-EGF-treated mice (Fig. 1); moreover, tumor growth in these 2 groups was not reduced when compared with that in control animals (Fig. 2). These data suggested that SHIN-3 cells might demand little or no extracellular growth factors to EGFR from host animals to grow. The facts that SHIN-3 cells secreted much TGFα and that TGFα did not promote cell growth in serum free cultures[3] might support these considerations.

Although 10^7 SHIN-3 cells formed tumors in anti-TGFα mAb-treated Sx animals (Fig. 1), anti-TGFα mAb administration caused a drastic reduction in tumor growth (Fig. 3). Ten μg of anti-TGFα mAb given to Sx animals 3 times a week significantly ($P < 0.05$) inhibited the tumor growth and growth inhibition was more by 20 μg of mAb (Fig. 3). This schedule of mAb injections was determined under the consideration of the clearance rate of antibodies in *nu/nu* mice (18). Moreover, as aggressive a tumor growth as that in Sx animals was resumed by the cessation of TGFα mAb administration (Fig. 3). Tumors grown in 7 groups of mice were excised after 6 weeks of SHIN-3 cell inoculation, and histological studies of these tumors were performed. Similar serous cystadenocarcinomas were observed in all 7 groups and representative histological findings in control, Sx, and Sx anti-TGFα mAb-treated animals were shown (Fig. 4). All these results suggested the important role of a TGFα/EGFR autocrine growth mechanism in this cell line *in vivo*.

The expression and the biological significance of a TGFα/EGFR autocrine mechanism were not limited to this cell line. We observed that 20 of 35 primary human ovarian cancers (57%) expressed TGFα/EGFR gene transcripts and proteins and that this autocrine mechanism played an important role in

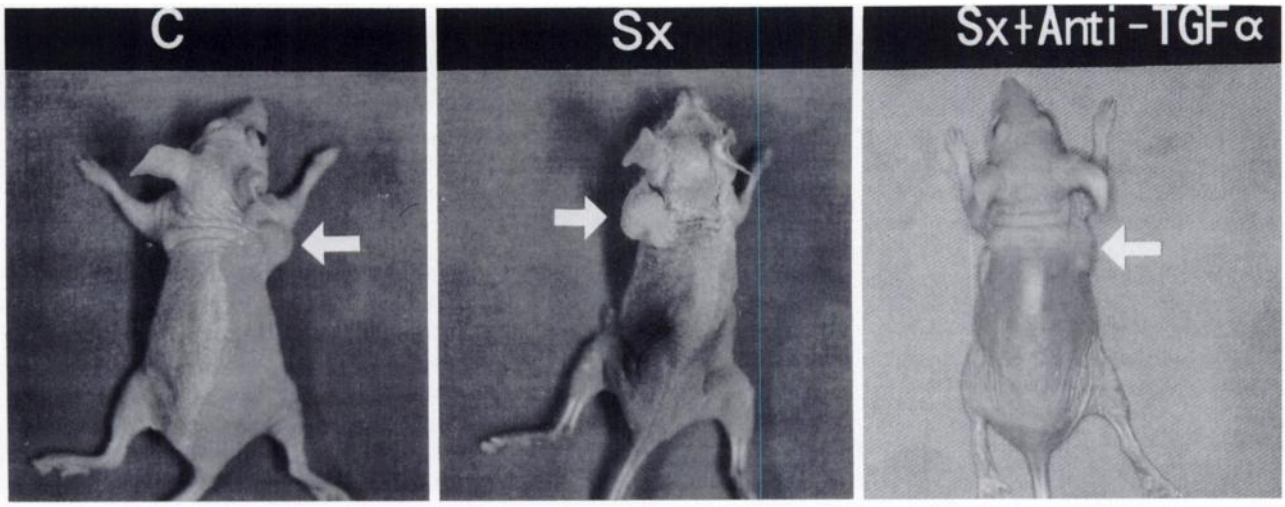

Fig. 1. SHIN-3 cell-inoculated *nu/nu* mice after 5 weeks of cancer cell inoculation. Ten million SHIN-3 cells were inoculated in sham-operated (control (*C*), sialoadenectomized (*Sx*), and Sx and anti-TGFα mAb-treated (*Sx+Anti-TGFα*) *nu/nu* mice. The smaller tumor in the *Sx+Anti-TGFα* mouse is noteworthy.

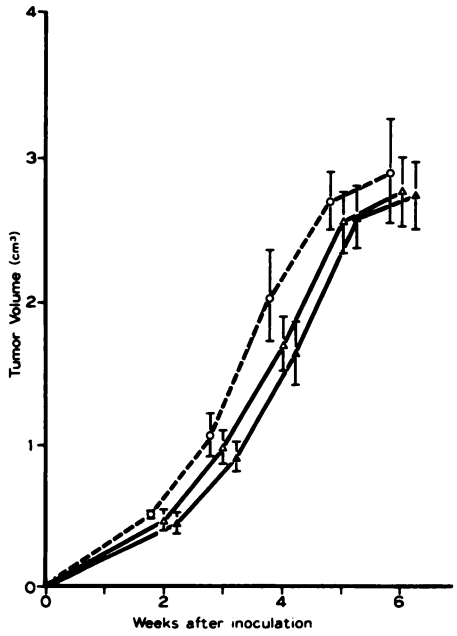

Fig. 2. Tumor growth curves in control, Sx, and Sx and anti-mouse EGF-treated *nu/nu* mice. Ten million SHIN-3 cells were inoculated into sham-operated (control) (○) ($n = 3$), Sx (△) ($n = 6$), and Sx and anti-mouse EGF-treated (▲) ($n = 3$) animals, and tumor sizes were measured twice a week. Tumor sizes were expressed in (length)×(width)²/2. The tumor growth curve of the Sx group represents the data in 6 animals of the following 3 groups: Sx and no further treatments were done ($n = 2$); Sx and nonimmune rabbit serum (100 μl/mouse) was injected daily ($n = 2$); and Sx and nonimmune mouse IgG (20 μg/mouse) was given 3 times a week ($n = 2$). No differences in tumor growth were observed among these 3 groups. All the treatments were started at the time of tumor cell inoculation and continued for 4 weeks. Data are the mean ± SE (*bars*).

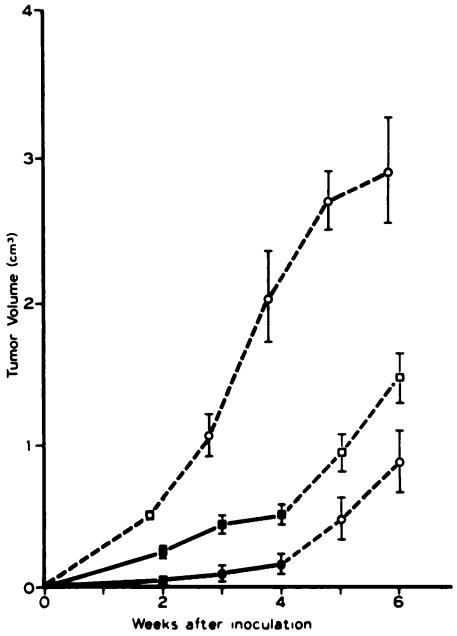

Fig. 3. Tumor growth curves in Sx and Sx and anti-TGFα mAb-treated *nu/nu* mice. Ten million SHIN-3 cells were inoculated into Sx (○) ($n = 6$ from 3 groups as in legend to Fig. 2), Sx and 10 μg of anti-TGFα mAb (■) ($n = 3$), and Sx and 20 μg of anti-TGFα mAb (●) ($n = 3$)-treated *nu/nu* mice. Anti-TGFα mAb injections (3 times a week) were started at the time of tumor cell inoculation and continued for 4 weeks. Data are the mean ± SE (*bars*). Tumor sizes in TGFα mAb-treated groups were smaller than in the Sx group at all points in the figure ($P < 0.05$).

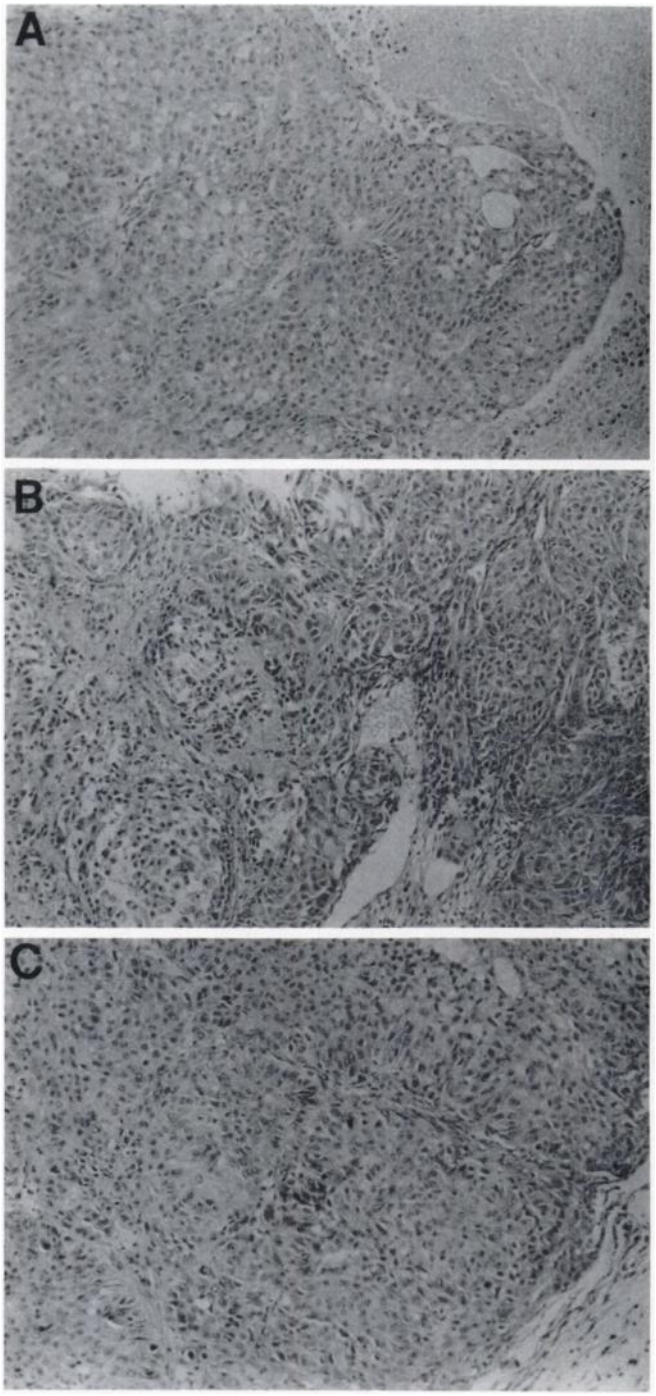

Fig. 4. Microphotographs of tumors excised from SHIN-3 cells inoculated *nu/nu* mice. Tumors were grown in control (*A*), Sx (*B*), and Sx and anti-TGFα mAb-treated (*C*) *nu/nu* mice. Tumors were excised after 6 weeks of SHIN-3 cell inoculation, fixed with 10% formaldehyde, sectioned, and stained. H & E, × 100.

cell growth *in vitro* (20) and *in vivo*.[4] We were much interested in the facts that SHIN-3 cells were transplantable and grown in Sx *nu/nu* mice as efficiently as in control animals but primarily ovarian cancers which expressed the TGFα/EGFR mechanism were not implantable to Sx *nu/nu* mice.[4] Although this cell line might not depend on extracellular growth factor(s) supplied from host animals as shown in this study, primary ovarian cancers were obviously dependent on it. It might be interpreted by these phenomena that established cell lines ac-

[4] Unpublished data.

quired the autocrine mechanism(s) better than primary cancer cells.

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