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# *IN VIVO* EVALUATION OF EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR $\beta_1$ IN MOUSE TUMOR MODELS

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**Summary**—The influence of modulating circulating levels of epidermal growth factor (EGF) and transforming growth factor $\beta_1$ (TGF-$\beta_1$) on tumor growth was examined in a variety of mouse models. Removal of the EGF-rich submandibular gland from host mice failed to alter the growth of a variety of human tumor xenografts or a C3H mouse tumor. Infusion of EGF from Alzet minipumps raised circulating EGF levels. However, only the A549 human tumor xenograft showed any significant increase in growth in the presence of EGF infusion and this response was marginal. The growth of Wehi 3BD+ and A549 tumor lines in culture was inhibited by TGF-$\beta_1$. The growth of these lines *in vivo*, however, was not significantly altered by the administration of TGF-$\beta_1$ via a variety of routes.

## INTRODUCTION

Growth factors, such as TGF-$\alpha$, TGF-$\beta$ and EGF, have been demonstrated to influence the proliferation of many different tumor types in the contrived conditions of culture [1, 2]. However, the regulatory role of these growth factors in normal or tumor cells in the *in vivo* environment is unclear. The administration to mice of monoclonal antibodies to the EGF-receptor indicate the growth of certain tumors *in vivo* are mediated via the EGF-receptor [3]. This is consistent with postulated models for autocrine and paracrine loops involving TGF-$\alpha$ [4]. Interestingly, it has also been suggested that EGF secreted from the submandibular gland may influence the growth of certain tumors [5].

In contrast to the stimulating actions of growth factors such as EGF and TGF-$\alpha$, the growth factor TGF-$\beta$ is inhibitory to certain tumor cell lines in culture [2]. Currently, no reports have described the *in vivo* activity of TGF-$\beta$ on tumors when administered distal to the tumor site. Twardzik *et al.* [6] have, however, described an inhibitory action of TGF-$\beta$ *in vivo* on A549 tumors when administered around the base of the tumor.

In order to gain a clearer understanding of the possible modulating actions growth factors can have on tumors *in vivo*, the present studies were performed. EGF was selected as a representative of stimulatory growth factors and TGF-$\beta_1$ as an inhibitory factor. The influence of modulating circulating levels of EGF and TGF-$\beta_1$ on tumor growth were examined in a variety of mouse models.

## MATERIALS AND METHODS

### *Animals*

Intact and sialoadenectomized athymic mice, Balb/c mice and (Balb/c $\times$ DBA/2)$F_1$ ($CDF_1$) mice were purchased from either Charles River Breeding Co. (Wilmington, Mass.) or Harlan Sprague–Dawley (Indianapolis, Ind.) and provided food and water *ad libitum*.

### *Tumors*

A549, A431 and MCF-7 cell lines were adopted to grow in athymic mice by s.c. inoculation of cultured cells. MCF-7 tumors were supplemented with 17-$\beta$-estradiol by implantation of a 1 cm silastic capsule, as described previously [7]. A C3H mouse bearing a spontaneous mammary tumor was obtained from the Frederick Cancer Center, Fredrick (Md). All tumors were passaged in athymic mice by sterile dissection of solid tumors and s.c. implantation of tumor fragments into the axillary region. Tumor size was determined by caliper measurement and weight estimated according to a published method [8]. Statistical comparisons were performed using the Gehan's generalized Wilcoxan

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test. Cultured WEHI 3BD+ cells were adapted to *in vivo* growth by i.p. inoculation and passed in Balb/c mice as an ascites line; all therapy studies were performed in $CDF_1$ mice.

*Culture*

WEHI 3BD+ cells were aseptically collected as ascites from a Balb/c mouse and cultured in DMEM media containing 10% fetal bovine serum and penicillin/streptomycin. Proliferation assays were performed by placing 5 ml of media containing $1 \times 10^4$ WEHI 3BD+ cells/ml in 15-ml sterile culture tubes. Following centrifugation, media was aspirated and 4.9 ml of media containing TGF-$\beta_1$ was added. Media was changed daily for 6 days. Respresentative tubes were collected on days 0, 2, 4 and 6 and stored frozen until assayed.

An A549 tumor was disaggregated with trypsin and the isolated cells cultured in McCoy's 5A media containing 10% fetal bovine serum and penicillin/streptomycin. Proliferation assays were performed by plating $1.5 \times 10^3$ A549 cells in 24 well dishes; 24 h after plating, media was aspirated and fresh media containing TGF-$\beta_1$ added (day 0). Media changes were performed daily and plates collected on days 0, 2, 4 and 6 and stored frozen until assayed. DNA was determined for A549 and Wehi 3BD+ studies as described previously [9].

*Growth factors*

Human recombinant TGF-$\beta_1$ was obtained from Oncogen Co., Bristol-Myers Squibb Co. (Seattle, Wash.) dissolved in 5 mM HCl. Just prior to use, the acid was neutralized with 10 × PBS containing either 10 mg/ml, bovine or mouse serum albumin (Sigma Chemical Co., St Louis, Mo.). For culture studies, the TGF-$\beta_1$ was subsequently serially diluted into media. For *in vivo* studies, TGF-$\beta_1$ was serially diluted in PBS containing 1 mg/ml serum albumin. Mouse derived EGF (Collaborative Research, Bedford, Mass.) was prepared in PBS and infused into mice from alzet minipumps (14-day release) by s.c. implantation on the back following anesthesia. Serum EGF levels were determined on 200 $\mu$l samples using a commercially available radioreceptor assay (Biomedial Technologies Inc., Stoughton, Mass).

## RESULTS

*Effects of sialoadenectomy*

The effect of removal of the EGF-rich submandibular gland (sialoadenectomy at least 10 days prior to use) from the host was examined on the growth of the human tumor xenografts MCF-7, A549 and A431 and a transplanted mammary tumor from a C3H mouse. Comparisons were made of tumor growth in intact vs sialoadenectomized athymic mice. In all studies with all tumor lines, however, the removal of the submandibular gland failed to significantly decrease the rate of tumor growth (see Table 1).

*EGF infusion*

Sample mice infused with different amounts of EGF (150, 30, 6 or 1.2 $\mu$g) from alzet minipumps (14-day release) were anesthetized and bled by cardiac puncture 7 days after implantation. Animals implanted with alzet pumps containing 150 $\mu$g of EGF (receiving 10.7 $\mu$g EGF/day) had higher circulating levels ($3.01 \pm 0.4$ ng/ml) than the control ($0.75 \pm 0.15$ ng/ml). Mice implanted with alzet pumps containing lesser amounts of EGF had levels very similar to control.

Table 1. Effect of sialoadenectomy of host mouse on tumor growth

| | | Median time to reach a 500 mg tumor size (days) | |
|---|---|---|---|
| Tumor line | Expt No. | Sialoadenectomized mice | Intact mice |
| MCF-7[a] | 1 | 35.5 | 39 |
| | 4 | 21.0 | 27 |
| A549 | 19 | 28.5 | 33.8 |
| | 20 | 21 | 20.5 |
| | 26 | 21.5 | 30.5 |
| | 27 | 23.5 | 22.8 |
| A431 | 1 | 18 | 20.3 |
| | 6 | 23.5 | 24.5 |
| Transplanted | 1 | 22.8 | 21 |
| C3H mouse tumor | 2 | 22 | 19.5 |

Tumor fragments were implanted, s.c., into either intact or sialoadenectomized athymic mice ($n = 10$). Tumor size was followed by weekly tumor measurements.

[a]These results represent mice treated with a 1 cm 17$\beta$-estradiol silastic capsule.

The effects of EGF infusion on the growth of tumor implants was subsequently examined. Fragments of either MCF-7, C3H, A431 or A549 tumors were implanted in athymic mice the same day as alzet pump implantations.

The infusion of EGF (up to 300 $\mu$g infused over 28 days) failed to stimulate any growth of the hormone-dependent MCF-7 tumor. This contrasted with sustained tumor growth produced by 17-$\beta$-estradiol treatment. The growth of the C3H tumors was not increased by EGF infusion at any of the dose levels tested (1.2, 6 and 30 $\mu$g over 14 days). A431 tumors on mice infused with EGF grew slightly more rapidly than tumors on control mice. However, the difference in time to reach a 500 mg median tumor size (MTS) between the control (24.5 days) and all other groups (including mice receiving 150 $\mu$g EGF; 20 days) was small and not statistically significant. In two experiments, A549 tumors on mice infused with EGF (0.24, 1.2, 6, 30 or 150 $\mu$g EGF over 14 days) grew slightly more rapidly than the control. However, the shortest time to reach a MTS of 500 mg in any of the EGF treated groups was only 7 days less than the control (27 days) and statistical significance was only observed between certain groups (1.2 and 150 $\mu$g EGF treated groups in expt 1 and 0.24 and 1.2 $\mu$g EGF treated groups in expt 2) and control. Furthermore, no dose relationship was observed in either study and one group (30 $\mu$g EGF, expt 2) grew slower than the control.

*Evaluation of TGF-$\beta_1$ as an antitumor agent*

In culture TGF-$\beta_1$ inhibited the proliferation of both the A549 and the mouse monomyelocytic leukemia line, WEHI 3BD+, in a dose-related manner (see Fig. 1). Concentrations of TGF-$\beta_1$ to cause 50% of the maximal inhibition achievable were similar for both tumor types (10–100 pg/ml). These studies were performed on cells sourced from *in vivo*.

Inoculation of WEHI 3BD+ into the i.p. cavity of CDF$_1$ mice produced a linear relationship between cell number inoculated and mouse lifespan over the titration examined ($10^6$–$10^3$). Inoculation of $10^5$ WEHI 3BD+ cells reproducibily gave a median survival time of approx. 15 days. Daily treatment with TGF-$\beta_1$ (i.p. days 1–9) with dose levels ranging from 28 to 0.06 $\mu$g/mouse/injection failed to increase survival time beyond that of the control (see Table 2). However, a dose-related decrease in body weight was observed with higher doses and early deaths occurred at the 28 and 14 $\mu$g/mouse/injection levels.

A549 tumors were grown as s.c. implants in the axillary region of athymic mice for 8 days and then distributed into groups of 10 mice matched with regard to median tumor size (20–40 mg). A preliminary study indicated i.v., s.c. and peritumoral injections of TGF-$\beta_1$ every 3 days for 5 doses did not produce a sustained inhibition of A549 tumor growth at the doses tested (see Table 3). However, with the schedule

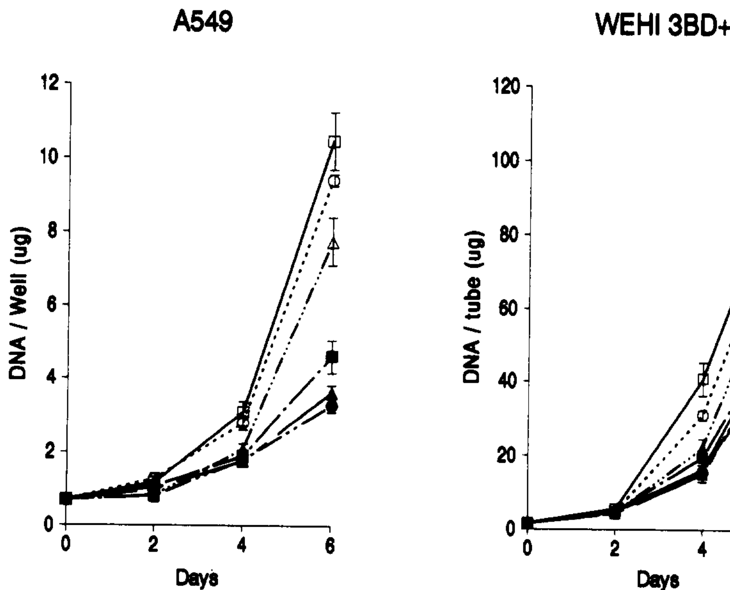


Fig. 1. Effect of TGF-$\beta_1$ on the growth of WEHI 3BD+ or A549 cells. Cells were incubated with 100 ng/ml (◇), 10 ng/ml (▲), 1 ng/ml (●), 100 pg/ml (■), 10 pg/ml (△), 1 pg/ml (○) TGF-$\beta_1$ or control (□). Media and TGF-$\beta_1$ were changed daily.

Table 2. Effect of TGF-$\beta_1$ on the median survival time (MST) of $CDF_1$ mice inoculated (i.p.) with $10^5$ WEHI 3BD+ cells

| Material | Dose ($\mu$g/mouse/inj.) | Total dose ($\mu$g/mouse) | Route and injection days | MST (days) | %T/C |
|---|---|---|---|---|---|
| Control | — | — | i.p. 1→9 | 15.5 | 100 |
| TGF-$\beta_1$ | 28 | 252 | i.p. 1→9 | 8/10 dead by day 10 | |
| | 14 | 126 | i.p. 1→9 | 14 | 90.3 |
| | 7 | 63 | i.p. 1→9 | 14 | 90.3 |
| | 3.5 | 31.5 | i.p. 1→9 | 14 | 90.3 |
| | 1.8 | 15.8 | i.p. 1→9 | 14 | 90.3 |
| | 0.9 | 7.9 | i.p. 1→9 | 14 | 90.3 |
| | 0.45 | 3.9 | i.p. 1→9 | 15 | 96.8 |
| | 0.22 | 2.0 | i.p. 1→9 | 15 | 96.8 |
| | 0.11 | 1.0 | i.p. 1→9 | 15 | 96.8 |
| | 0.06 | 0.5 | i.p. 1→9 | 14.5 | 93.5 |

[10 mice/group; %T/C = MST of treated group divided by the control MST × 100].

used, the maximum tolerated dose was only reached with the i.v. route.

## DISCUSSION

The present set of studies have examined the influence of modulating circulating growth factor levels in host mice on the growth of implanted tumors. In contrast to a previous report [5], we observed no difference in the growth rate of a transplanted C3H mouse mammary tumor when implanted in either intact or sialoadenectomized athymic mice. The reason for the differing results is unclear at present. The lack of influence sialodencetomy had on the C3H mouse tumor is, however, consistent with our findings for a variety of humor xenografts and for a carcinogen-induced rat mammary tumor previously reported [10].

EGF infusion has been reported to increase the rate of growth of certain EGF-receptor hyperproducing tumor cells when implanted in athymic mice [11]. In our hands, EGF infusion sufficient to quadruple control circulating levels produced, at best, only a marginal stimulation of A549 tumor growth, and little to no effect on A431, MCF-7 and C3H tumors. The report [12] that EGF can stimulate MCF-7 tumor growth only demonstrated a transient effect following a short duration of treatment. Our present studies indicate no sustained growth is produced even with prolonged EGF infusion. Overall, the insensitivity of these transplanted tumors to EGF modulation suggest either the changes in circulating EGF levels were insufficient or the tumors are insensitive to EGF *in vivo*.

Both the A549 and WEHI 3BD+ lines we are currently using in *in vivo* were demonstrated to be inhibited by TGF-$\beta_1$ when studied in culture. Our findings for these cell lines are consistent with previous reports in the literature [2, 13]. When examined *in vivo*, TGF-$\beta_1$ failed to increase the lifespan of $CDF_1$ mice implanted with WEHI 3BD+ cells, although a maximal

Table 3. Effect of TGF-$\beta_1$ on A549 tumor growth in athymic mice

| Material | Dose ($\mu$g/mouse/inj.) | Total dose ($\mu$g/mouse) | Injection days and route | Median time to 500 mg tumors (days) | T-C days |
|---|---|---|---|---|---|
| Control | — | — | 8, 11, 14, 17, 20[a] | 29.75 | 0 |
| TGF-$\beta_1$ | 45 | 225 | 8, 11, 14, 17, 20 s.c. | 31.8 | 2.1 |
| | 22.5 | 112.5 | 8, 11, 14, 17, 20 s.c. | 29.8 | 0.1 |
| | 5.6 | 28 | 8, 11, 14, 17, 20 s.c. | 27.5 | −2.3 |
| | 1.4 | 7 | 8, 11, 14, 17, 20 s.c. | 25 | −4.8 |
| | 0.35 | 1.8 | 8, 11, 14, 17, 20 s.c. | 24 | −5.8 |
| TGF-$\beta_1$ | 22.5 | 112.5 | 8, 11, 14, 17, 20 p.t. | 29.3 | 0.6 |
| | 5.6 | 28 | 8, 11, 14, 17, 20 p.t. | 25.5 | −4.3 |
| | 1.4 | 7 | 8, 11, 14, 17, 20 p.t. | 34.8 | 5.1 |
| | 0.35 | 1.8 | 8, 11, 14, 17, 20 p.t. | 25.8 | −4.1 |
| | 0.09 | 0.45 | 8, 11, 14, 17, 20 p.t. | 32.3 | 2.6 |
| TGF-$\beta_1$ | 320 | 1600 | 8, 11, 14, 17, 20 i.v. | 7/10 dead by day 29 | |
| | 80 | 400 | 8, 11, 14, 17, 20 i.v. | 29 | −0.8 |
| | 20 | 100 | 8, 11, 14, 17, 20 i.v. | 27 | −2.8 |
| | 5 | 25 | 8, 11, 14, 17, 20 i.v. | 28.3 | −1.5 |
| | 1.25 | 5 | 8, 11, 14, 17, 20 i.v. | 28.3 | −1.5 |
| Mitomycin C | 3 mg/kg | | 8, 14, 20, i.p. | >56 | >26.25 |

[a] Separate controls for s.c., peritumoral (p.t.) and i.v. did not differ significantly and were combined ($n$ = 30). Only mitomycin C treatment produced a statistically significant difference in time to reach 500 mg MTS ($P < 0.05$).

tolerated dose was reached and a very wide range of doses were tested. Furthermore, in a preliminary study, no clear activity for TGF-$\beta_1$ was observed on A549 tumor growth in athymic mice following either i.v., s.c. or peritumoral injections. The TGF-$\beta_1$ dose administered i.v. was sufficient to reach the maximal tolerated level, however, s.c. and peritumoral treatments did not reach lethality. Our peritumoral doses of TGF-$\beta_1$ did, nevertheless, range from the doses reported [6] to produce a marked tumor inhibition to approx. 100 times greater.

Overall, these *in vivo* studies indicate it is difficult to alter tumor proliferation via the exogenous administration of peptide growth factors. In the manner evaluated, we observed no meaningful tumor stimulation with EGF and no antitumor activity using TGF-$\beta_1$. Further studies will examine TGF-$\beta_1$ using different treatment schedules and tumor types.

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