NEOPLASTIC PROGRESSION OF RAT TRACHEAL EPITHELIAL CELLS INVOLVES RESISTANCE TO TRANSFORMING GROWTH FACTOR BETA

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Abstract — Primary, transformed, and tumor-derived rat tracheal epithelial (RTE) cells were grown in serum-free medium containing 0 to 300 pg/mL transforming growth factor beta (TGFβ). TGFβ markedly inhibited the growth of primary RTE cells with a 50% drop in the efficiency of colony formation seen at TGFβ concentrations between 10 and 30 pg/mL. The effect of TGFβ on preneoplastic RTE cells was similar to the effect on normal primary RTE cells. Cell lines established from tumors produced by inoculation of transformed RTE cells into nude mice were relatively resistant to TGFβ-induced growth inhibition. Resistance to TGFβ-induced growth inhibition, therefore, appears to be a late event in the development of neoplasia.

TGFβ is a multifunctional cellular regulator first recognized by its ability to interact with TGFα or epidermal growth factor and confer the property of anchorage independent growth to the normal rat kidney fibroblast clone, 49F. In rodent fibroblasts, anchorage independent growth is a property of transformed cells, that, in rodent systems, is frequently associated with tumorigenicity, and hence the name of this growth factor. TGFβ is the first described member of an entire family of homologous regulatory peptides.[1] In recent years, the number of diverse functions ascribed to this peptide have increased markedly,[1] but regulated cell growth during wound healing and embryogenesis are apparently important in vivo roles of this multifunctional peptide. Altered responsiveness to TGFβ appears to play a role in the development of neoplasia since TGFβ inhibits the growth of most, if not all, primary epithelial cells, while a number of TGFβ-resistant transformed or carcinoma cell lines have been reported.

The purpose of the studies described here is to determine (1) whether resistance to TGFβ-induced growth inhibition is associated with neoplastic progression of RTE cells and (2) the stage in neoplastic development at which this occurs. We have previously reported the preliminary results of these studies (1986-87 Annual Report, LMF-120, pp. 518-521).

METHODS

To determine the effect of TGFβ on different stages in neoplastic progression, we classified RTE cells into 4 classes: (1) primary RTE cells were normal cells isolated directly from animals; (2) preneoplastic RTE cells were nontumorigenic cell lines previously isolated from primary RTE cells transformed by n-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or X rays; (3) tumorigenic RTE cells were also cell lines previously isolated from primary RTE cells transformed by MNNG or X rays. However, these cell lines produced tumors when inoculated into nude mice; and (4) tumor-derived RTE cells were cell lines isolated from tumors that appeared in nude mice inoculated with tumorigenic RTE cells.

Primary RTE cells were harvested from male, 6-8 wk old, F344/N rats raised in the Institute as previously described.[2] Transformed RTE cells were periodically tested for Mycoplasma and consistently found negative (Microbiological Associates).

For TGFβ testing, cells were plated at clonal density (250-2500 cells/dish) in a modified serum-free medium[2] without cholera toxin (CT), because of the antagonistic effect of cyclic AMP inducers on TGFβ, and with 0.05% bovine serum albumin (BSA), since higher concentrations of BSA are toxic in the absence of CT. Two days after plating, the cells were refed with the same medium containing 0, 1, 3, 10, 30, 100, or 300 pg/mL TGFβ. Primary RTE cells were fixed and stained 10 days after plating. Transformed and tumor-derived RTE cells were fixed and stained 7 days after plating due to differences in inherent growth rate. Colonies of greater than 30 cells were enumerated.

The percent colony-forming efficiency (CFE) was calculated by dividing the number of colonies in each petri dish by the number of cells plated and multiplying by 100. Relative colony-forming efficiency (relative CFE) was calculated by dividing the CFE in each treatment group by the CFE in the control (0 pg/mL) group.

RTE cell lines were inoculated into nude mice to classify the lines as preneoplastic or tumorigenic. Cells were inoculated into the subcutaneous tissue of the axilla of weanling nude mice at the same or similar passage to TGFβ testing to determine if these cells were transformed and nonneoplastic (designated as preneoplastic) or transformed and neoplastic (designated as tumorigenic) at the time of testing. Two tumor-derived cell lines were inoculated as positive controls. Masses appearing in the nude mice were measured weekly. Nude mice were euthanized when tumor size reached 1.5 cm^2.

Number of colonies formed rather than relative CFE were used in the statistical analyses to prevent any inadvertent biasing of the analysis during standardization of the data. The log of the number of colonies formed was fit as a linear function of the log of the TGFβ concentration using as an offset the log of the plating density. The slope and intercept were obtained for each assay as a separate estimate. The TGFβ sensitivity of the primary, preneoplastic, tumorigenic, and tumor-derived cells were compared using a generalized linear model for Poisson-distributed data as a function of the log of the TGFβ concentration. The analysis was performed assuming that the individual slopes were drawn from a random sample of cell lines within the classification of primary, preneoplastic, tumorigenic, or tumor derived.

To facilitate visual comparison between cell lines, all figures in the text compare values of cell lines using relative CFE rather than raw colony counts.

RESULTS

TGFβ was a powerful inhibitor of primary RTE cell growth with a 50% reduction in CFE seen at TGFβ concentrations between 10 and 30 pg/mL (Fig. 1). The effect of TGFβ on 7 preneoplastic RTE cell lines was similar to the effect on primary RTE cells (Fig. 2).

In contrast, tumorigenic RTE cell lines were less sensitive to TGFβ than were primary RTE cell lines (Fig. 3). While 1 of the 5 cell lines showed a positive interaction of plating density and CFE that prevented inclusion in either the statistical analyses or figures, 3 of the 4 remaining tumorigenic cell lines had increased resistance to TGFβ-induced growth inhibition relative to the primary cells. For 2 of these tumorigenic cell lines, so many colonies formed at TGFβ concentrations $\geq$ 30 pg/mL that accurate determination of CFE could not be made. However, as judged by their ability to produce massive numbers of colonies in the presence of TGFβ, these tumorigenic cell lines were clearly TGFβ resistant. One cell line; EGV 118, AIG3; formed a tumor but was extremely sensitive to TGFβ (relative CFE = 0.03 at 300 pg TGFβ/mL).

To determine if EGV 118, AIG 3 represented a bona fide exception to the observations made here that preneoplastic RTE cell lines were TGFβ-sensitive while tumorigenic cell lines were TGFβ-resistant, two experiments were performed. First, a cell line; EGV 118, AIG 3T; was isolated

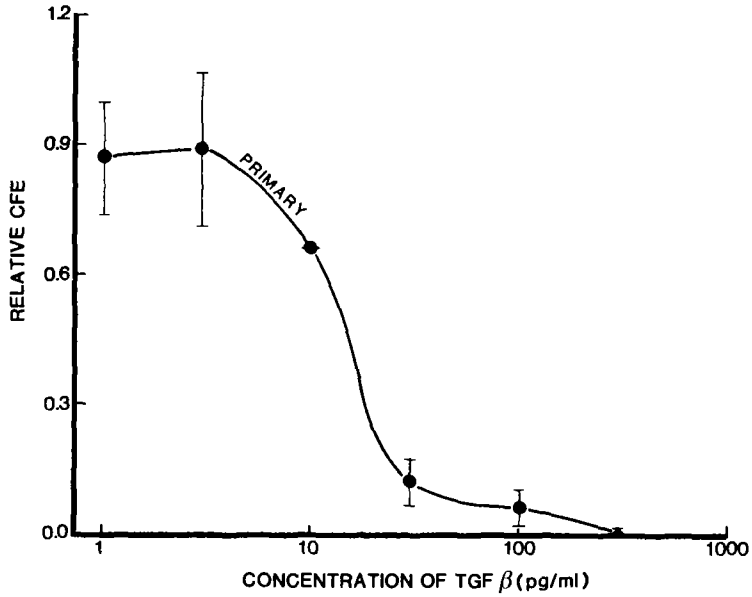

Figure 1. The effect of TGFβ on primary rat tracheal epithelial cells. Petri dishes were plated with 2500 primary rat tracheal epithelial cells/dish in serum-free medium. Two days after plating, medium was switched to serum-free medium containing 0, 1, 3, 10, 30, 100, or 300 pg/mL TGFβ. Dishes were fixed and stained 10 days after plating and colonies of 30 cells or more were enumerated. Points represent the mean of 2 experiments ± the standard error of the mean.

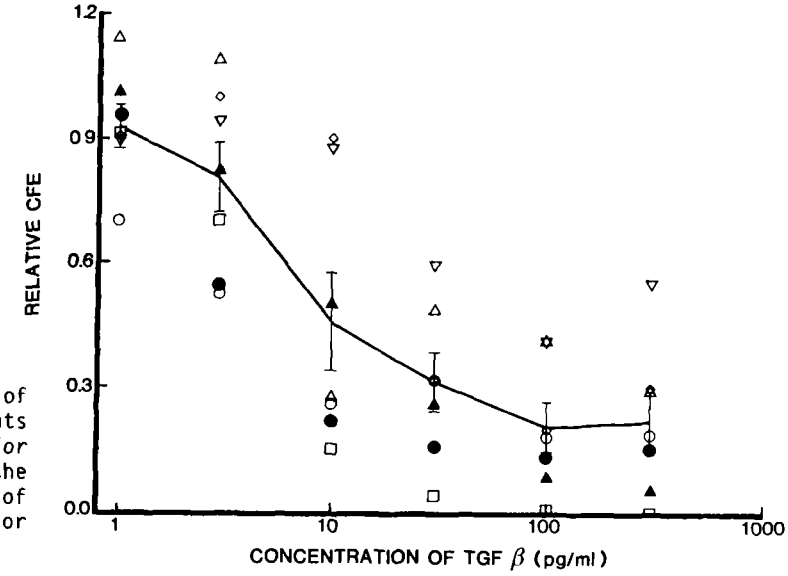

Figure 2. TGFβ sensitivity of preneoplastic cell lines. Points represent the mean of 3 dishes for each cell line. The mean ± the standard error of the mean of all of these cell lines is included for comparison.

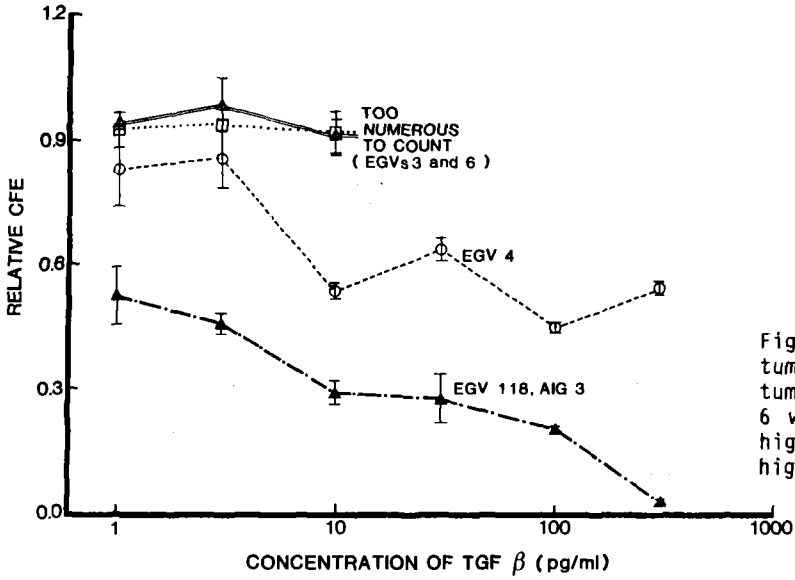

Figure 3. The effect of TGFβ on tumorigenic cell lines. Two tumorigenic cell lines, EGV 3 and EGV 6 were too numerous to count at the higher plating densities used for the higher TGFβ concentrations.

from the tumor produced by inoculating EGF 118, AIG 3 into nude mice. EGV 118, AIG 3T was tested for responsiveness to TGFβ and was TGFβ resistant (Fig. 4). Second, the sensitivity of the nude mouse inoculation assay to detect rare tumorigenic cells was determined. Variable numbers of tumorigenic EGV 6 cells were injected with preneoplastic EGV 9 cells to give a constant inoculum of 10^6 cells. As few as 10 out of 10^6 cells were capable of producing a tumor (Table 1).

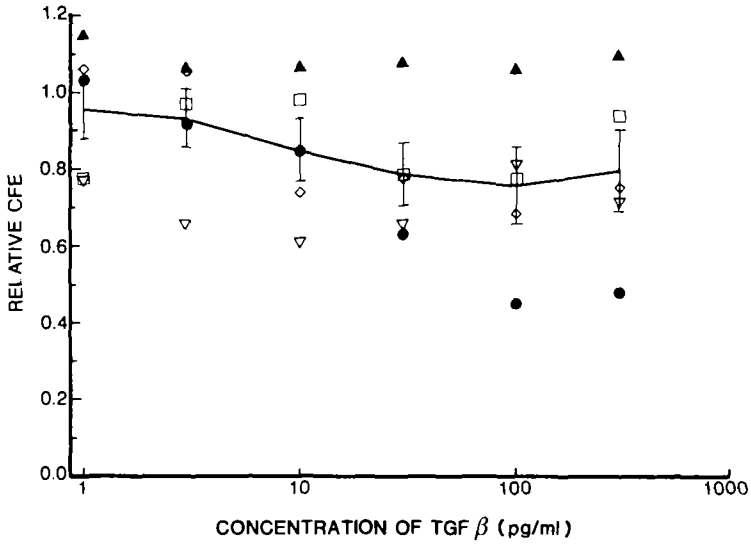

Figure 4. The effect of TGFβ on 5 tumor cell lines. Points represent the mean of 3 to 5 dishes for each cell line. The mean ± the standard error of the mean of these tumor-derived cells is included for reference.

Table 1
Number of Neoplastic RTE Cells Needed to
Produce a Tumor in the Nude Mouse Assay[a]

No. of EGV 6 Cells	Animals with Tumors	Tumors/ Injection
10^6	2/2	4/4
10^5	2/3	4/6
10^4	5/5	10/10
10^3	5/5	9/10
10^2	5/5	10/10
10^1	4/5	5/10
0	0/30	0/60

[a]The most tumorigenic of the transformed RTE cells studied in the first series of nude mouse inoculations was EGV 6. EGV 6 was mixed with EGV 9 to produce a total of 10^6 cells for inoculation into nude mice. The number of tumors produced per inoculation site and the number of tumors per animal are listed in the table. Ten EGV 6 cells, as calculated by serial dilution, were sufficiently to produce tumors in 5 of 10 inoculation sites.

Because the tumor-derived cell lines had undergone further selection by the nude mouse, they were included as a separate group. All 5 tumor-derived cell lines were TGFβ resistant (Fig. 4). Because tumor-derived cell lines sometimes have higher CFEs than either preneoplastic or primary RTE cells, we investigated the effect of plating density on the TGFβ response of three of the

tumor-derived cell lines using three different plating densities for each cell line. A plating range of 250 to 1500 cells did not affect the TGFβ response of these cell lines (data not shown).

Using a generalized linear model for Poisson-distributed data as a linear function of the log of the TGFβ1 concentration, the slopes of the tumor-derived cells were significantly smaller than those of either primary cells or preneoplastic cell line ($p \leq 0.01$) but not statistically different from those of the tumorigenic cells ($p > 0.05$). The slopes of the TGFβ responses of the tumorigenic cells were significantly smaller than the slopes of the primary cells ($p \leq 0.05$) but not statistically different from those of the preneoplastic or tumor-derived cells ($p > 0.05$). Differences between the primary cells and the preneoplastic cells were not statistically significant ($p > 0.05$). The calculated slopes and standard errors for each cell line are shown in Table 2.

DISCUSSION

The TGFβ responsiveness of RTE cells at different stages of progression to neoplasia was determined. The nontumorigenic RTE cell lines used in this study represent cells at a preneoplastic stage of progression to neoplasia. In this study, these preneoplastic cells responded to the endogenous growth inhibitor TGFβ in a manner similar to normal primary RTE cells. Tumorigenic RTE cells were variable in their response to TGFβ. Statistically, the response of these cells could only be separated from the response of primary cells. Cells isolated from tumors were resistant to TGFβ-induced growth inhibition. A summary graph of the effects of TGFβ on normal, preneoplastic and tumor-derived RTE cells is shown in Figure 5.

We interpreted the variability in the response of the tumorigenic cell lines as being associated with potentially variable numbers of neoplastic cells since we also showed that 10 or fewer highly neoplastic RTE cells mixed with 10^6 nonneoplastic RTE cells could produce a tumor. In support of this interpretation, the newly isolated tumor-derived cell line EGV 118, AIG 31 was resistant to TGFβ (mean relative CFE at 300 pg/mL TGFβ varied from 0.56 to 0.75 for the three different assays), while the parent cell line was the only tumorigenic cell line clearly sensitive to TGFβ (mean relative CFE at 300 pg/mL TGFβ = 0.03). Although the large inoculum (1 to 2 x 10^6) used to test for tumorigenicity could have contained small numbers of neoplastic cells, those cells would not have been present in the small inocula used for TGFβ assays. Thus, a tumorigenic cell line could potentially be TGFβ sensitive if the majority of the cells were, in fact, nontumorigenic.

Growth inhibition and terminal differentiation have previously been reported as effects of TGFβ on normal airway epithelial cells isolated from man and rabbits[5,6] as has the failure to respond to TGFβ in some cell lines derived from lung tumors.[5,6] This study differs from the previous studies by including a broad group of preneoplastic cell lines in conjunction with nude mouse inoculations to define the effect of TGFβ on cells at different stages of progression to neoplasia. We conclude that resistance to TGFβ plays a role as a late event in neoplastic progression of RTE cells.

Table 2

Tumorigenicity of Cell Lines Relative to TGFβ Sensitivity and Stage in Neoplastic Progression

Cell Line	Slope ± SEM of the TGFβ Response Curve[a]	Tumors/Injection
Primary Cells		
1st Experiment	-0.5318 ± 0.0748	N/D[b]
2nd Experiment	-0.4644 ± 0.0353	N/D
Preneoplastic Cell Lines		
EGV 1, Clone 19	-0.2328 ± 0.0406	0/6
EGV 9	-0.9513 ± 0.0281	0/6
EGV 9, AIG 1	-0.2405 ± 0.0130	0/6
EGV 201	-0.4949 ± 0.0137	0/6
EGV 203	-0.2771 ± 0.0644	0/6
EGV 205	-0.1248 ± 0.0116	0/6
EGV 207	-0.3297 ± 0.0390	0/6
Tumorigenic Cell Lines		
EGV 3	-0.0150 ± 0.0286	2/6
EGV 4	-0.1006 ± 0.0114	6/6
EGV 6	-0.0034 ± 0.0214	6/6
EGV 118, AIG 3	-0.3238 ± 0.0119	1/6
EGV 204	N/D[c]	1/6
Tumor-Derived Cell Lines[d]		
EGV 4T2	+0.1721 ± 0.0068	5/6
EGV 6T	-0.0028 ± 0.0121	5/6
EGV 3T	+0.0047 ± 0.0240	N/D
EGV 118, AIG 3T	-0.0763 ± 0.0127	N/D
EGV 204T	-0.1541 ± 0.0109	N/D

[a]The slope of the TGFβ response was calculated from the log-log plot (log of the number of colonies formed plotted against the log of the TGFβ concentration using the log of the plating density as an offset).

[b]N/D = Not done. In previous experiments, we have never obtained tumors in nude mice inoculated with primary RTE cells.

[c]The slope to the TGFβ response of EGV 204 could not accurately be calculated due to an interaction with cell density (see text).

[d]Tumor-derived cell lines were obtained from the tumors appearing in nude mice inoculated with the tumorigenic cell lines.

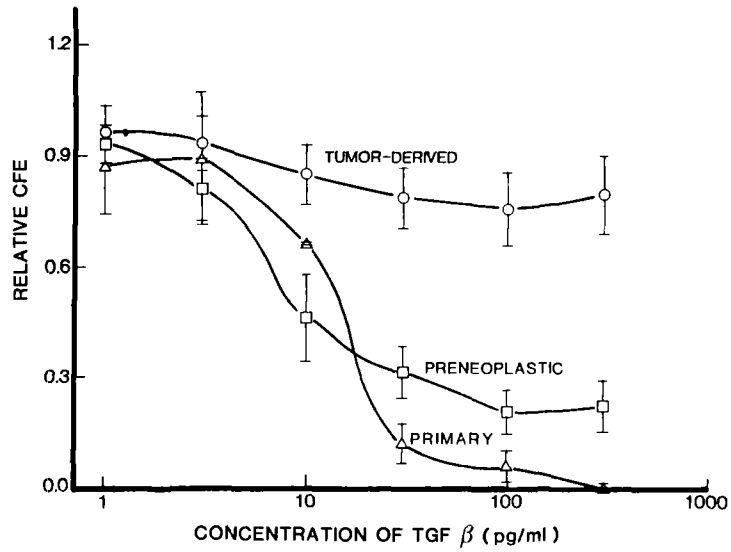

Figure 5. The effect of TGFβ on normal, preneoplastic, and tumor-derived rat tracheal epithelial cells. The overall effect of TGFβ on normal, preneoplastic, and normal rat tracheal epithelial cells shows a general tendency for primary rat tracheal epithelial cell and preneoplastic rat tracheal epithelial cells to remain sensitive to TGFβ-induced growth inhibition. Conversely, neoplastic (tumor-derived) rat tracheal epithelial cells were less sensitive to TGFβ-induced growth inhibition.

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