Involvement of pRB Family in TGFβ-dependent Epithelial Cell Hypertrophy

Harold A. Franch,* Jerry W. Shay,‡ Robert J. Alpern,* and Patricia A. Preisig*

Departments of *Internal Medicine and ‡Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75235-8856

Abstract. Although renal hypertrophy is often associated with the progressive loss of renal function, the mechanism of hypertrophy is poorly understood. In both primary cultures of rabbit proximal tubules and NRK-52E cells (a renal epithelial cell line), transforming growth factor β1 (TGFβ) converted epidermal growth factor (EGF)-induced hyperplasia into hypertrophy. TGFβ did not affect EGF-induced increases in c-fos mRNA abundance or cyclin E protein abundance, but inhibited EGF-induced entry into S, G_2, and M phases. EGF alone increased the amount of hyperphosphorylated (inactive) pRB; TGFβ blocked EGF-induced pRB phosphorylation, maintaining pRB in the active form. To determine the importance of active pRB in TGFβ-induced hypertrophy, NRK-52E cells were infected with SV40 large T antigen (which inactivates pRB and related proteins and p53), HPV16 E6 (which degrades p53), HPV16 E7 (which binds and inactivates pRB and related proteins), or both HPV16 E6 and E7. In SV40 large T antigen expressing clones, the magnitude of EGF + TGFβ-induced hypertrophy was inhibited and was inversely related to the magnitude of SV40 large T antigen expression. In the HPV16-infected cells, EGF + TGFβ-induced hypertrophy was inhibited in E7- and E6E7-expressing, but not E6-expressing cells. These results suggest a requirement for active pRB in the development of EGF + TGFβ-induced renal epithelial cell hypertrophy. We suggest a model of renal cell hypertrophy mediated by EGF-induced entry into the cell cycle with TGFβ-induced blockade at G_1/S, the latter due to maintained activity of pRB or a related protein.

RENAL tubular hypertrophy occurs in a number of conditions, including diabetes mellitus, loss of renal mass, protein feeding, chronic metabolic acidosis, and potassium deficiency (13). In many of these conditions the hypertrophy has been postulated to cause progressive loss of renal function (6, 53). Despite its importance, the mechanism by which tubular hypertrophy occurs is largely unknown. One possible mechanism is that hypertrophy represents an aborted cell cycle, with cells entering the G_1 phase and initiating protein synthesis and growth, but failing to progress into S phase. This hypothesis would predict that events associated with entrance into G_1 would be the same in hyperplastic and hypertrophic processes, but that events associated with the transition between G_1 and S phases would be different, with only hyperplastic cells progressing into S phase.

pRB, the product of the retinoblastoma gene, plays a key role in regulating the G_1/S transition (2, 7, 21). Active (hypophosphorylated) pRB inhibits G_1/S progression; hyperphosphorylation inactivates pRB, allowing progression into S phase. Transforming growth factor β (TGFβ) exerts an antiproliferative effect in a number of cells (36), which is mediated in part by inhibition of pRB phosphorylation (maintenance of active pRB) and blockade of the cell cycle at G_1/S (32). In addition, TGFβ has been implicated in renal tubular and vascular smooth muscle cell hypertrophy (17, 42, 46, 52). In the present study, a model of hypertrophy in renal proximal tubule epithelial cells was developed to test the hypothesis that TGFβ-mediated hypertrophy is coupled to cell cycle processes. Primary cultures of rabbit proximal tubule cells developed hyperplasia in response to epidermal growth factor (EGF), hypoplasia in response to TGFβ1, and hypertrophy in response to the combination of EGF + TGFβ. The ability of TGFβ to convert EGF-induced hyperplasia to hypertrophy was related to inhibition to pRB phosphorylation and the maintenance of active pRB or a related protein.

Materials and Methods

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), except as indicated below. Dulbecco's modified Eagle's medium and Ham's F12 culture media, fetal bovine serum, and trypsin/EDTA were from GIBCO BRL (Gaithersburg, MD); transferrin was from Miles Pentex (Kankakee, IL); penicillin and streptomycin were from BioWhitaker, Inc., M. A. Bioproducts (Walkersville, MD); type 1 collagenase was from Boehringer Mannheim Corp. (Indianapolis, IN); culture dishes were from Corning Glass-

Address all correspondence to Patricia Preisig, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8856. Tel.: (214) 648-8628. Fax: (214) 648-9100.

works (Corning, NY); recombinant human TGFβ1 and EGF were from R&D System (Minneapolis, MN); Hoechst H33258 was from Calbiochem (La Jolla, CA); [^{3}H]thymidine, [^{3}H]phenylalanine, and the ECL kit were from Amersham (Arlington Heights, IL); pRB immunoaffinity purification kit and anti-pRB antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Primary rabbit proximal tubule cultures were prepared as previously described (22). Briefly, 4–6-wk old New Zealand white rabbits were killed and renal cortex trimmed from the kidneys, sliced with a Stadie-Riggs tissue slicer, and incubated in 0.1% type 1 collagenase for 40 min at 37°C with moderate shaking. The resulting tissue suspension was centrifuged on an isosmotic 50% percoll gradient at 20,000 *g* for 30 min (JA-20 rotor and J2-21M centrifuge; Beckman Instrs., Fullerton, CA). Tubules aspirated from the F4 fraction were resuspended and centrifuged to wash out the percoll. The pellet was then diluted by culture media to achieve a concentration of 3,000 tubules/ml and inoculated onto tissue culture dishes. Culture media consisted of a 1:1 mixture of DME and Ham's F12 supplemented with 5 μg/ml insulin, 50 nM hydrocortisone, 35 μg/ml transferrin, 29 nM Na selenite, 20 μM ethanolamine, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. 3% fetal bovine serum was added to the medium for the first 3 d to facilitate cell attachment. Cells were grown to ~80% confluence and then rendered quiescent by the removal of insulin and hydrocortisone for 48 h before initiating the experimental protocols.

NRK-52E cells (a rat kidney epithelial cell line) were obtained from the American Type Culture Collection (Rockville, MD) at passage 15, and passaged and grown in low glucose DME with 5% FCS. These cells were infected with a retroviral construct containing the SV40 large T antigen gene with tsA58 and U19 mutations, and a neomycin resistance gene, both inserted into the vector pZIPSV(X)I (26, 27). For infection with HPV16 E6 and E7, the retroviral construct consisted of HPV16 E6 and/or E7 genes and a neomycin resistance gene, both inserted into the vector PLXSN (24, 48). Control cells were infected with the vector containing only the neomycin resistance gene. These constructs were infected into an amphotropic packaging cell line PA317. The packaging cells were allowed to reach confluency, the medium was removed, and fresh medium (10% FBS DME) placed on the PA317 cells for 16 h. The media was then removed from the packaging cells, filtered through a 0.4-μm filter, and placed on NRK-52E cells when 20% confluent. The cells were exposed to the virus for 10 h, the viral media replaced with 5% CS DME, and selection begun 48 h later with 400 μg/ml G-418. After 10 d the G-418 dose was reduced to 200 μg/ml for maintenance. G-418 was removed at the time cells were plated for studies. SV40 large T antigen clonal cell lines were established by limiting dilution. Cells plated for studies were rendered quiescent by the removal of serum for 48 h before initiating the experimental protocols.

Mice transgenic for SV40 large T antigen (Tg[SV40E]Bri7) (1) were killed by decapitation and the kidneys were removed, washed in ice cold PBS, decapsulated, and microdissected. Approximately 1 mm of mouse proximal convoluted tubule (MPCT),[1] mouse proximal straight tubule (MPST), and inner stripe of the outer medullary collecting duct (MMCD1 and MMCD2) were washed in PBS and separately cultured in 24-well plates with a 1:1 mixture of Ham's F12 and low glucose DME supplemented with 1.5 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum. Cells plated for studies (passages 5–20) were rendered quiescent at 50% confluence for 48 h before initiating the experimental protocols.

To determine SV40 large T antigen expression, Western blotting was used. Cells were grown to confluence in 100-mm dishes, rinsed in PBS × 2, and lysed at 4°C in 1 ml of RIPA buffer (1% NP-40, 0.4% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0) containing 2 mM EDTA, 0.1 mg/ml PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. The lysate was centrifuged at 12,000 *g* for 10 min at 4°C and the supernatant stored at −70°C. Equal amounts of protein, determined by Lowry (34), were loaded on a 6% polyacrylamide gel. Western blotting was performed as described below for pRB, using a 1:100 dilution of primary antibody (1:1 mixture of two monoclonal antibodies Pab416 and Pab419 [20]). SV40 large T antigen expression was quantitated by scanning densitometry. Results are expressed as percent expression compared to the highest expressing clone.

1. *Abbreviations used in this paper*: MMCD, mouse medullary collecting duct; MPCT, mouse proximal convoluted tubule; MPST, mouse proximal straight tubule.

In general, all studies compared four groups: (*a*) control (with both EGF and TGFβ1 vehicles); (*b*) EGF, with TGFβ1 vehicle; (*c*) TGFβ1, with EGF vehicle; and (*d*) the combination of EGF and TGFβ1. Recombinant human TGFβ1 was reconstituted in 4 mM HCl containing 0.1% heat-treated BSA. Recombinant human EGF was reconstituted in PBS. Media were changed daily. In all studies, except those examining the dose response to TGFβ1 or EGF, TGFβ1 was used at 10^{-10} M and EGF at 10^{-8} M.

Measurement of Cell Protein, DNA, and Cell Size

Primary cultures were grown in 6-well dishes and cell lines in 12-well dishes, washed with PBS, harvested with 0.25% trypsin and 1 mM EDTA for 30 min (primary cultures) or 0.05% trypsin and 0.5 mM EDTA for 5 min (cell lines), pelleted at 1,500 *g* for 5 min, and washed again with PBS. The final pellet was resuspended in 1 ml lysis buffer (50 mM Na_2PO_4, pH 7.4) and the cells lysed on ice by repeated passage though a 27-gauge needle. The lysate was then aliquoted and stored at −70°C for protein and DNA determination. The aliquots frozen for measuring DNA content contained 1 mM EDTA. Protein was measured by Lowry (34). DNA was measured using the fluorescent compound Hoechst H33258 in an SLM 8000C fluorimeter (30). Cell hypertrophy was defined as an increase in the ratio of protein/DNA, determined in aliquots obtained from the same well.

Cell size was obtained by measuring forward light scatter on an EPICS II profile analyzer. Cells were harvested and suspended in 0.25% trypsin/1 mM EDTA. Forward light scatter was measured on 8,000 to 12,000 cells, after gating to remove cellular debris.

Measurement of [^{3}H]Thymidine and [^{3}H]Phenylalanine Incorporation

Rates of DNA and protein synthesis were measured as rates of [^{3}H]thymidine and [^{3}H]phenylalanine incorporation, respectively. To measure the rate of phenylalanine incorporation, 2 μCi/well of [^{3}H]phenylalanine were added 8 h before harvest of cells grown in 12-well tissue culture plates. To harvest, cells were washed with PBS and protein precipitated with 0.5 ml/well of 5% TCA. After washing with distilled water to remove all unincorporated labeled phenylalanine, proteins were solubilized with 0.5 ml of 0.5 N NaOH/0.1% Triton X-100 per well, and the resulting protein suspension was counted in a scintillation counter (LS 3801; Beckman). Parallel wells for each experimental group were harvested and suspended in 0.25% trypsin/1 mM EDTA, and cells counted with a hemocytometer. Results are expressed as [^{3}H]phenylalanine uptake/cell (cpm/cell).

To measure the rate of thymidine incorporation, 1 μCi/well of [^{3}H]thymidine was added 6 h before harvest of cells grown in a 96-well tissue culture plate. Cells were harvested onto filter paper using a PHD cell harvester (Cambridge Technologies, Cambridge, MA), and filters counted in a scintillation counter. Parallel wells for each experimental group were harvested and suspended in 0.25% trypsin/1 mM EDTA and cells counted with a hemocytometer. Results are expressed as [^{3}H]thymidine uptake/cell (cpm/cell).

Retinoblastoma (pRB) Protein Phosphorylation

The proportion of pRB in the hypo- or hyperphosphorylated form was determined on SDS-PAGE by mobility shift (15, 32). On Western blots, the hypophosphorylated species is detected as an apparent 105-kD protein and the hyperphosphorylated species as an apparent 110–116-kD protein. Cells were rinsed with PBS, harvested on ice with cold lysis buffer (pRB immunoaffinity purification kit, supplemented with 0.1 mg/ml PMSF, 0.66 U/ml aprotinin, 1 mM sodium orthovanadate, and 2 μg/ml leupeptin) by scraping with a rubber policeman, lysed by repeated passage through a 27-gauge needle, and centrifuged at 15,000 *g* for 20 min at 4°C. The supernatant was incubated with agarose-conjugated mouse monoclonal pRB antibodies for 6 h. Bound protein was then eluted with pH 11.2 elution buffer (pRB immunoaffinity purification kit) and stored at −70°C for Western blotting. Samples were mixed with loading buffer (1% SDS, 10% glycerol, 1% β-mercaptoethanol, 5% electrophoretic buffer), boiled for 5 min, electrophoresed on a 6% polyacrylamide gel, electrophoretically transferred to nitrocellulose, and blotted using a polyclonal anti-pRB antibody at 1:50 dilution (31). pRB bands were detected using ECL and exposed on Kodak X-Omat film for 3–15 s. The percentage of pRB in the hyperphosphorylated form was quantitated by subtracting the amount of pRB in the hypophosphorylated state from the local amount of pRB, using scanning densitometry. Results are expressed as the percent of total pRB in the hyperphosphorylated (ppRB) form.

Cyclin E Protein Abundance

To measure cyclin E protein abundance samples were size fractionated on a 8.75% polyacrylamide gel, and Western blotting performed with a 1:3,000 dilution of primary antibody (rabbit anti-cyclin E) (a generous gift from Dr. James Roberts, Fred Hutchinson Cancer Institute, Seattle, WA). Cyclin E bands were detected using ECL and abundance quantitated by scanning densitometry.

c-fos mRNA Abundance

Total cellular RNA was extracted using a modification of the method of Chirgwin et al. (3, 37). Cultured cells were scraped in guanidium thiocyanate solution (4 M guanidium thiocyanate, 0.5% *N*-lauroylsarcosine, 0.1 M 2- mercaptoethanol, and 25 mM sodium citrate [pH 7.0]), disrupted by gentle aspiration through a 22-gauge needle twice, centrifuged through a 5.7 M CsCl cushion at 52,000 rpm for 2 h at 20°C (Optima TLX: TL55 rotor; Beckman Instruments), and further purified by ethanol precipitation. Total RNA was size fractionated by agarose-formaldehyde gel electrophoresis and transferred to nylon filters (Genescreen-Plus; New England Nuclear, Boston, MA) in 20× SSC. Prehybridization, hybridization, washing, and exposure to film were performed as described previously (3, 37). Radiolabeled probes were made by random primer using full-length c-fos and GAPDH cDNAs (5, 14). c- fos and GAPDH abundance were quantitated by scanning densitometry.

Cell Cycle Analysis

Cells grown in 6-well culture dishes were harvested with 0.25% trypsin/0.5 mM EDTA, washed in PBS × 1 on ice, pelleted by centrifugation at 1,500 *g* for 5 min at 4°C, and resuspended in 0.5 ml saline GM (8.0 gm/L NaCl, 1.1 gm/L glucose, 0.4 gm/L KCl, 0.29 gm/L $NaH_2PO_4 \cdot 7H_2O$, 0.15 mg/L KH_2PO_4, 0.5 mM EDTA, pH 7.4). While vortexing gently, 1.5 ml ethanol was added slowly, and the cells were allowed to fix overnight at 4°C in containers wrapped tightly in foil (4, 45). The cells were then pelleted by centrifugation, resuspended in 250 μl of 1 mg/ml RNase A, allowed to sit for 30 min at 37°C, repelleted by centrifugation, and resuspended in 200 μl of 50 μg/ml propidium iodide. Cells were analyzed on a FACscan (Becton Dickinson, Mountain View, CA) using Lysis II software. Cell cycle phase analysis was done using the CellFIT program (Becton Dickinson).

Statistics

All data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA. Linear regression analysis was used to correlate SV40 large T antigen expression with the magnitude of hypertrophy.

Results

EGF Induces Hyperplasia and TGFβ1 Induces Hypoplasia

Initial studies were performed on rabbit proximal tubule cells grown to ~80% confluence, rendered quiescent for 48 h (see Materials and Methods), and then exposed to EGF (10^{-8} M), TGFβ1 (10^{-10} M), the combination of EGF (10^{-8} M) + TGFβ1 (10^{-10} M), or vehicles for 48–96 h. Fig. 1 shows the effect of these compounds on DNA content/well. EGF alone increased DNA content/well at all time points, consistent with a mitogenic effect. TGFβ1 alone decreased DNA content/well at all time points, consistent with an antiproliferative effect. At 48 and 72 h the combination of EGF + TGFβ1 had little effect on DNA content/well, but at 96 h DNA content/well was decreased.

To confirm that the changes in DNA content/well represented changes in cell proliferation, and not cell death, [^{3}H]thymidine incorporation was measured. As shown in Fig. 2, EGF consistently increased, and TGFβ1 consistently decreased thymidine incorporation, confirming their hyperplastic and hypoplastic effects, respectively. The combination of EGF + TGFβ1 increased thymidine incorporation at 24 h, reflecting a predominant EGF effect, had no effect on thymidine incorporation at 48 h, and decreased thymidine incorporation at 72 h, reflecting a predominant TGFβ1 effect. Since these cells are not entirely quiescent in the absence of serum (indicated by a basal rate of thymidine incorporation) the apparent lag time in the TGFβ1 effect on thymidine incorporation is probably due to the lack of synchronization of the cell population.

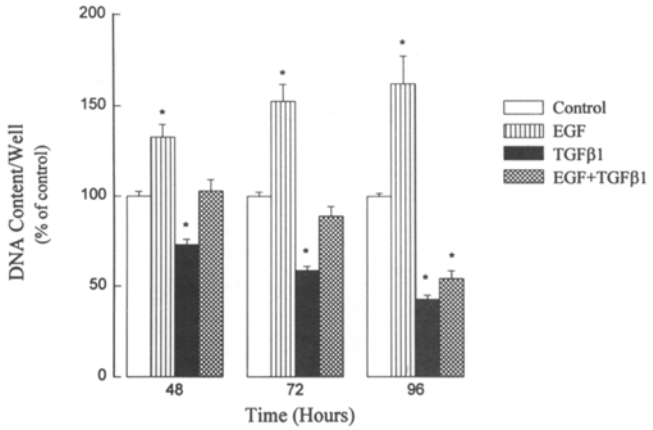


Figure 1. Effect of EGF and/or TGFβ1 on DNA content/well. Primary cultures of rabbit proximal tubule cells were exposed to EGF (10^{-8} M) and/or TGFβ1 (10^{-10} M) and/or vehicle for the indicated times. DNA content/well is plotted on the y-axis as a percent of control values (cells treated with vehicles only). n = 18 at 48 h, n = 24 at 72 and 96 h. * = $P < 0.01$ vs. control.

EGF + TGFβ1 Induces Cell Hypertrophy

To determine whether these models were associated with cell hypertrophy, cell protein was measured, and the ratio of protein/DNA calculated. As shown in Fig. 3, EGF alone had no effect on the ratio of protein/DNA, TGFβ1 alone induced a small increase in the ratio of protein/DNA, and the combina-

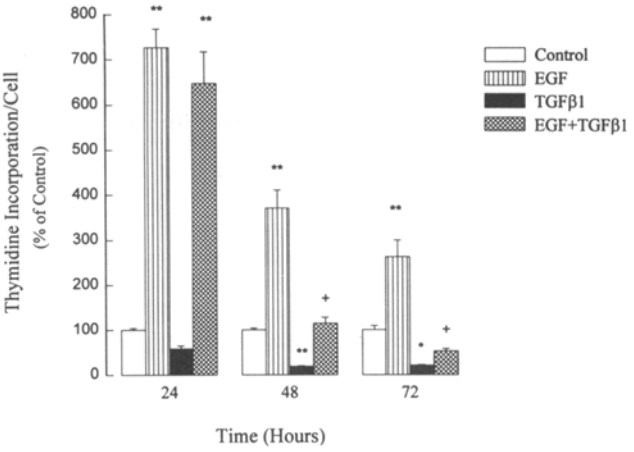


Figure 2. Effect of EGF and/or TGFβ1 on [^{3}H]thymidine incorporation/cell. Cells were grown and treated as in Fig. 1. Thymidine incorporation is plotted on the y-axis as a percent of control values (cells treated with vehicles only). n = 48 at 24 and 48 h, n = 36 at 72 h. * = $P < 0.05$ vs. control, ** = $P < 0.01$ vs. control; + = $P < 0.01$ vs. EGF alone.

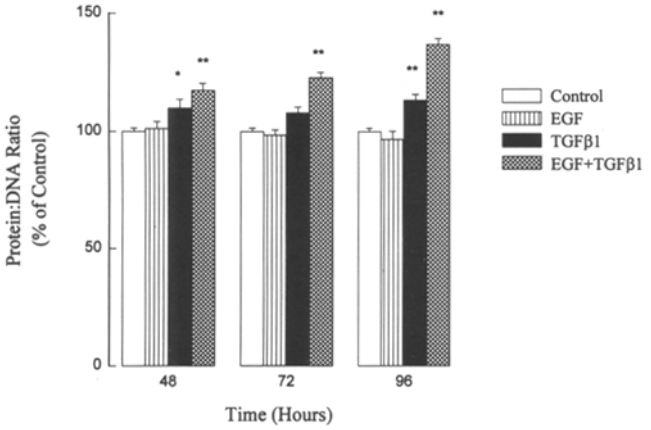


Figure 3. Effect of EGF and/or TGFβ1 on the ratio of protein/DNA. Cells were grown and treated as in Fig. 1. The ratio of protein/DNA is plotted on the y-axis as a percent of control values (cells treated with vehicles only). n = 18 at 48 h, n = 24 at 72 and 96 h. * = $P < 0.05$ vs. control; ** = $P < 0.01$ vs. control.

tion of EGF + TGFβ1 caused a progressive increase in the protein/DNA ratio. Similar results were observed in NRK-52E cells, which also hypertrophy following exposure to EGF + TGFβ1 (ratio of protein/DNA increased by 44 and 77% at 48 and 96 h, respectively) (data not shown).

To examine the dose dependence of the hypertrophic effect of EGF + TGFβ1, studies were performed in which TGFβ1 concentration was varied between 10^{-12} and 10^{-10} M while EGF concentration was held constant at 10^{-8} M, and EGF concentration was varied between 10^{-9} and 10^{-7} M while TGFβ1 concentration was held at 10^{-10} M. When TGFβ1 concentration was varied, the hypertrophic effect of TGFβ1 was not seen with 10^{-12} M TGFβ1, a 6% increase in the ratio of protein/DNA was seen with 10^{-11} M TGFβ1, and a 21% increase was seen with 10^{-10} M TGFβ1. In contrast, when EGF concentration was varied, similar increases in the ratio of protein/DNA were observed with EGF concentrations ranging from 10^{-9} to 10^{-7} M. All further studies utilized 10^{-10} M TGFβ1 and 10^{-8} M EGF.

The above effect of EGF + TGFβ1 on the ratio of protein/DNA could be due to an effect on extracellular matrix protein accumulation, and not reflect a true increase in cell size (hypertrophy). To address this possibility flow cytometry was used to measure mean forward light scatter, an index of cell size. Fig. 4 *a*, illustrates a representative experiment plotting cell number as a function of forward light scatter at 72 h. The data was summarized in Fig. 4 *b*. EGF alone had a small effect on mean forward light scatter between 48 and 96 h, and TGFβ1 alone had no effect. However, the combination of EGF + TGFβ1 significantly increased mean forward light scatter at all time points. These studies confirm that the combination of EGF + TGFβ1 is hypertrophic in proximal tubule cells. The small increase in mean forward light scatter seen with EGF alone is consistent with an increased number of cells in G_1, S, and G_2 phases as a consequence of EGF-induced proliferation. With TGFβ1 alone the small increase in the protein/DNA ratio with no effect on cell size is probably secondary to TGFβ1-induced stimulation of extracellular matrix protein production, an effect that would be included

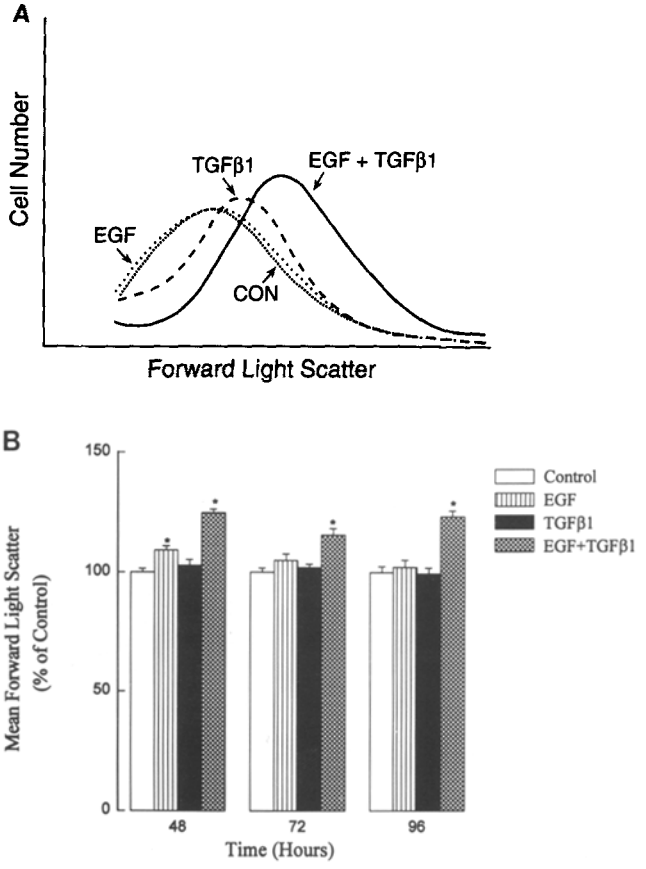


Figure 4. Effect of EGF and/or TGFβ1 on mean forward light scatter. Cells were grown and treated as in Fig. 1. (*A*) Representative tracing of relative cell number (plotted on the y-axis) as a function of forward light scatter (plotted on the x-axis) at 72 h. (*B*) Mean forward light scatter is plotted on the y-axis as a percent of control values (cells treated with vehicles only). n = 15 per group. * = $P < 0.01$ vs. control.

in the protein/DNA, but not the forward light scatter measurement.

To examine whether the increase in cell size was associated with an increase in protein synthesis, [^{3}H]phenylalanine incorporation was measured. Consistent with a mitogenic effect (parallel increases in DNA and protein content), EGF alone increased phenylalanine incorporation (Fig. 5). TGFβ1 alone had no effect on phenylalanine incorporation. The combination of EGF + TGFβ1 profoundly increased phenylalanine incorporation. Thus, hypertrophy in the latter group was accompanied by an increase in protein synthesis. Taken together these studies demonstrate that in rabbit proximal tubule cells EGF alone is hyperplastic, TGFβ1 alone is hypoplastic, and the combination of EGF + TGFβ1 is hypertrophic.

EGF + TGFβ-induced Hypertrophy Involves G_1 Arrest

To determine the cell cycle phase of the hypertrophied cells propidium iodide labeling of NRK-52E cells was assessed on the FACScan following 48 h exposure to EGF and/or TGFβ1. As shown in Fig. 6, EGF alone increased, while TGFβ1 alone decreased the percentage of cells in S/G_2/M phases. TGFβ1

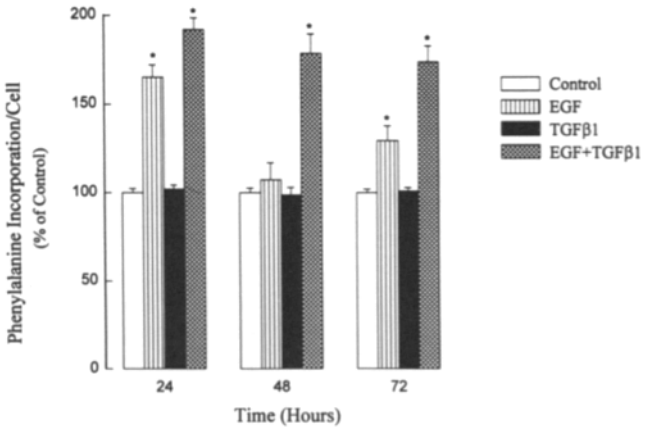


Figure 5. Effect of EGF and/or TGFβ1 on [^{3}H]phenylalanine incorporation/cell. Cells were grown and treated as in Fig. 1. Phenylalanine incorporation is plotted on the y-axis as a percent of control values (cells treated with vehicles only). $n = 18$ per group. * = $P < 0.01$ vs. control.

significantly inhibited EGF-induced entrance into S/G_2/M. Similar results were found in primary cultures of proximal tubule cells (data not shown). Thus, in both cell types, EGF induced a shift into the S/G_2/M phases, which was blocked by TGFβ.

Failure to enter S/G_2/M phases in the TGFβ-treated cells could be due to arrest in G_1 or failure to enter G_1. To address this the effect of TGFβ1 on two markers of EGF-induced cell cycle entry was examined. As shown in Fig. 7, EGF alone induced a transient increase in c-fos mRNA abundance, that peaked at 15–30 min. TGFβ1 addition did not affect the c-fos increase, supporting the fact that TGFβ1 does not inhibit EGF-induced entrance into G_1.

The next study examined the effect of EGF and/or TGFβ1 on cyclin E protein abundance, that typically begins to rise

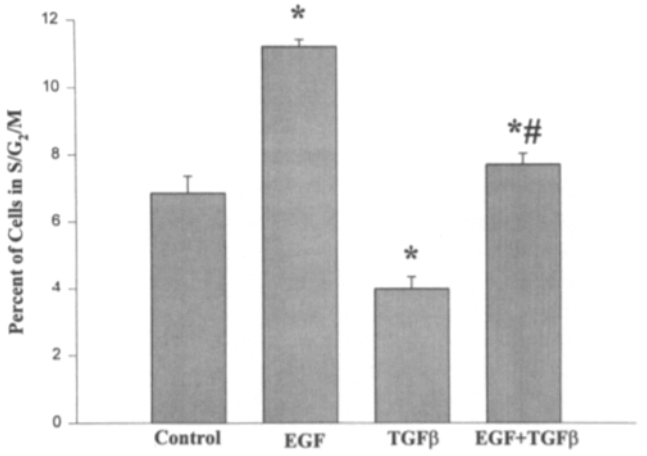


Figure 6. Effect of EGF and/or TGFβ1 on the percent of cells in S/G_2/M phases of the cell cycle. NRK-52E cells were rendered quiescent for 48 h and then treated with EGF (10^{-8} M) and/or TGFβ1 (10^{-10} M) for 48 h before harvest. Cell cycle phase analysis was performed as described in the Methods section. * = $P < 0.01$ vs. control; # = $P < 0.01$ vs. EGF alone.

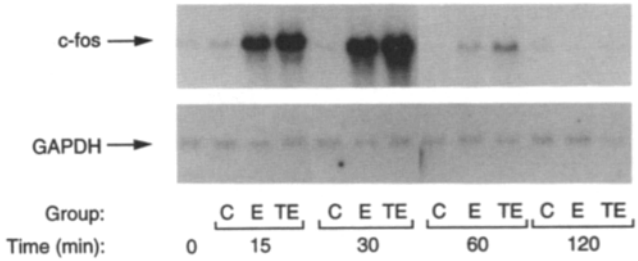


Figure 7. Effect of EGF and EGF + TGFβ1 on c-fos mRNA abundance. Northern blot. NRK-52E cells were rendered quiescent for 48 h, and then exposed to vehicle alone (*C*), EGF (10^{-8} M) (*E*), or EGF (10^{-8} M) + TGFβ1 (10^{-10} M) (*TE*) for the indicated times.

in mid-G_1. Fig. 8 *a*, shows a Western blot at 48 h, while *b* summarizes the results of four experiments in NRK-52E cells. Cyclin E protein abundance was increased following exposure to EGF. The combination of EGF + TGFβ1 increased cyclin E protein abundance to an extent similar to that seen with EGF alone, again suggesting that TGFβ does not prevent entrance into G_1. Taken together these results suggest that TGFβ-mediated hypertrophy is associated with cells entering G_1 but not progressing to S phase.

TGFβ1 Inhibits EGF-induced pRB Phosphorylation

The next studies examined whether regulation of pRB phosphorylation contributed to EGF + TGFβ-induced hypertrophy. In its hypophosphorylated state pRB is active, inhibiting progression from G_1 to S phase, while hyperphosphorylated pRB is inactive, allowing progression to S phase (33, 39, 49). As described in Materials and Methods the inactive, hyperphosphorylated form of pRB migrates more slowly on protein gel electrophoresis than the active, hypophosphorylated form (15, 32). As shown in Fig. 9, in primary cultures of proximal tubule cells EGF alone induced a time-dependent increase in pRB phosphorylation when added to quiescent cells, which was apparent as early as 6 h, and became maximal at 12 h. This allows cells to progress into S phase and undergo hyperplasia.

To determine if the ability of TGFβ1 to convert EGF-induced hyperplasia to hypertrophy involves regulation of pRB phosphorylation, we examined the effect of TGFβ1 on EGF-induced pRB phosphorylation. Fig. 10 *a*, shows a Western blot at 48 h, while *b* summarizes results of three experiments at 24–72 h in primary cultures of proximal tubule cells. EGF alone induced hyperphosphorylation, and TGFβ1 induced hypophosphorylation, although this latter effect did not reach statistical significance. TGFβ1 inhibited EGF-induced phosphorylation of pRB at 48 and 72 h. The lack of effect at 24 h corresponds to the failure of TGFβ1 to inhibit the EGF-induced increase in thymidine incorporation at 24 h in primary cultures (Fig. 2).

TGFβ-mediated Hypertrophy Is Dependent on an Active Member of the pRB Family

The above studies demonstrate that TGFβ1-induced maintenance of active, hypophosphorylated pRB correlates with TGFβ1's ability to convert EGF-induced hyperplasia to hypertrophy. If pRB plays a key role in this process, it would

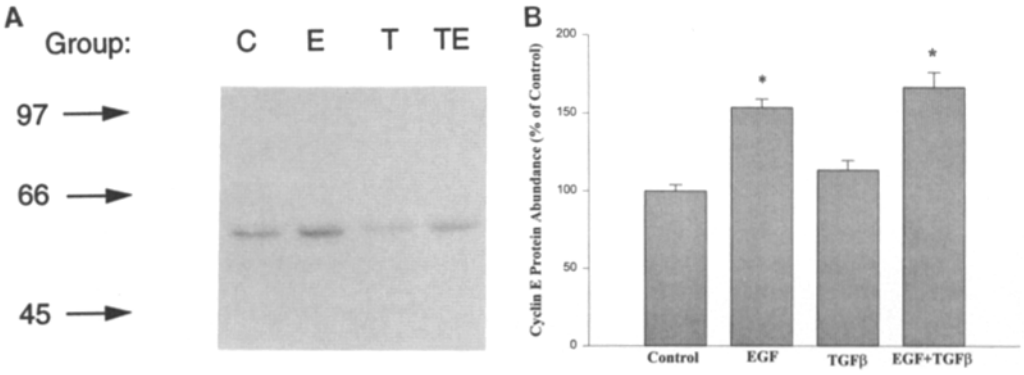


Figure 8. Regulation of cyclin E abundance by EGF and TGFβ1. (*A*) Western blot on NRK-52E cells. Cells were grown and treated as in Fig. 6 with vehicle alone (*C*), EGF alone (*E*), TGFβ1 alone (*T*), or EGF + TGFβ1 (*TE*). (*B*) Effect on EGF and TGFβ1 on cyclin E abundance. The percentage change in cyclin E abundance is plotted on the y-axis.

be predicted that prior inhibition of pRB would prevent the development of hypertrophy following exposure to EGF + TGFβ1.

For these studies two approaches were used. First, we generated several renal cell lines from renal tubule segments microdissected from mice transgenic for the SV40 large T antigen (Tg[SV40E]Bri7) (1). SV40 large T antigen binds to the hypophosphorylated, active form of pRB and two other pRB-related proteins (p107 and p130), effectively sequestering the proteins and rendering them inactive (11, 19, 35). In these lines, SV40 large T antigen expression was variable (Fig. 11, *solid bars*). Therefore, we examined the correlation between SV40 large T antigen expression and EGF + TGFβ1-induced hypertrophy. As shown in Fig. 11 there was an inverse relationship between expression of SV40 large T antigen (*solid bars*) and the magnitude of the EGF + TGFβ1-induced hypertrophy (*open bars*). The two cell lines with highest large T antigen expression and least hypertrophy were both of proximal tubule origin, while the cell lines with lowest large T antigen expression and significant hypertrophy were of collecting tubule origin. Thus, it was possible that the key variable that determined whether cells developed hypertrophy was cell type rather than large T antigen expression.

As a second approach we overexpressed SV40 large T antigen, which was introduced into NRK-52E cells by retroviral infection (see Materials and Methods). We then generated a number of clonal cell lines stably expressing the vector containing the SV40 large T antigen (pZIPNeoSV(X)I). Fig. 12 *a* is a Western blot demonstrating the variability of SV40 large T antigen expression in 10 clones infected with the pZIPNeoSV(X)I vector. Six of these clones (lanes *1*, *4–7*, and *10*), representing the range of SV40 large T antigen expression, were selected for further study. Fig. 12 *b* compares the magnitude of hypertrophy as a function of SV40 large T antigen expression in these clones, compared to EGF + TGFβ1-induced hypertrophy in cells infected with the vector without SV40 large T antigen insert (lane *11* in *a*; *open circle* in *b*). As can be seen, SV40 large T antigen expression inhibits hypertrophy, and there is an inverse relationship between the magnitude of SV40 large T antigen expression and the degree of EGF + TGFβ1-induced hypertrophy. As shown by linear regression, the y-intercept is significantly less than 100%, demonstrating that a small amount of SV40 large T antigen expression has a large effect on the development of hypertrophy. These results support the hypothesis that active pRB or a related family member plays a key role in the development of EGF + TGFβ1-induced hypertrophy.

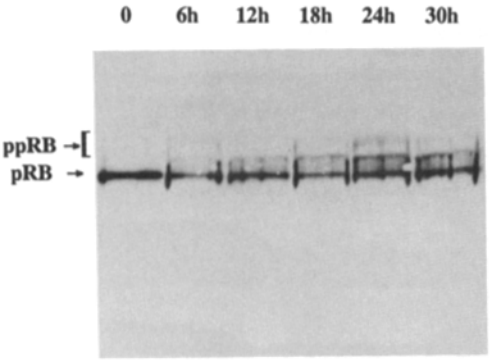


Figure 9. Time course of the effect of EGF on pRB phosphorylation. Western blot on primary cultures of proximal tubule cells. pRB is the hypophosphorylated (active) species of pRB; ppRB is the hyperphosphorylated (inactive) species of pRB.

SV40 large T antigen inactivates pRB, pRB-related proteins, and p53. To confirm that the modulation of hypertrophy was secondary to inactivation of a member of the pRB family, and not an effect on p53, NRK-52E cells were infected with retroviruses containing either HPV16 E6 (which degrades p53), HPV16 E7 (which binds and effectively sequesters pRB and pRB-related proteins), or both (8, 24, 48). A similar approach has been used in keratinocytes to demonstrate that TGFβ-mediated inhibition of growth involves regulation of prB activity (44). EGF + TGFβ1-induced hypertrophy was inhibited in E7 and E6E7 expressing cells, but not in the E6 expressing cells, as assessed by protein/DNA ratio (Fig. 13). Similar results were obtained when cell size was assessed (Fig. 14). These studies provide additional support for a role for active pRB or a related family member in TGFβ-dependent hypertrophy.

In the studies in which the family of pRB proteins was inactivated, either by SV40 large T antigen or HPV16 E7, inhibition of hypertrophy was associated with induction of hyperplasia, assayed as DNA content per well (data not shown). This observation provides additional support for a hypertrophy model in which the development of hypertrophy is the consequence of arresting a hyperplastic process.

Discussion

Renal hypertrophy occurs in a number of conditions, some of which are associated with the development of sclerosis

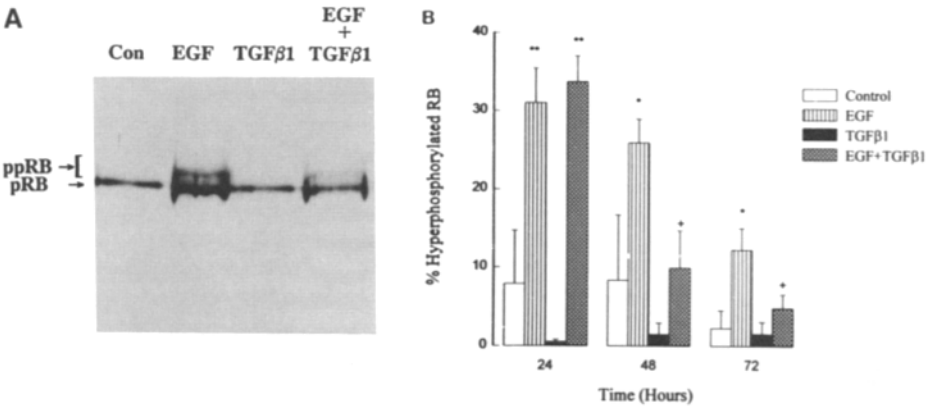


Figure 10. Regulation of pRB phosphorylation by EGF and TGFβ1. (*A*) Western blot on primary cultures of proximal tubule cells. pRB and ppRB defined as in Fig. 9. (*B*) Effect of EGF and TGFβ1 on the percent of pRB in the hyperphosphorylated state. Cells were grown and treated as in Fig. 1. The percent of pRB in the hyperphosphorylated state is plotted on the y-axis. See Materials and Methods for method of quantitating pRB. $n = 3$. * = $P < 0.05$ vs. control; ** = $P < 0.01$ vs. control; + = $P < 0.05$ vs. EGF alone.

and the progressive loss of renal function. It has been postulated that the hypertrophic process, while possibly beneficial initially, elicits responses that over the long term contribute to renal destruction (6, 53). Similarly, while cardiac hypertrophy is in some respects beneficial, it too has deleterious effects, and is postulated to contribute long term to loss of cardiac function (9, 51). In spite of its potential importance in renal and cardiovascular disease, the cellular and molecular mechanisms responsible for hypertrophy are poorly understood. An understanding of these mechanisms can provide an initial basis for examining the processes by which hypertrophy may cause deleterious effects on renal and cardiac function.

One possible mechanism for the development of hypertrophy is for cells to enter the growth cycle, but not progress into S phase. This would result in the synthesis of new cell

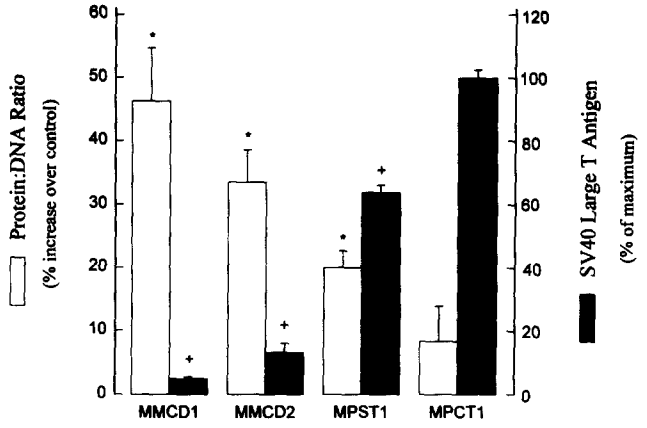
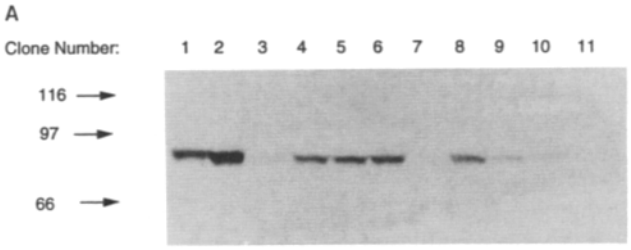


Figure 11. SV40 large T antigen expression and the magnitude of the EGF + TGFβ1-induced hypertrophy in cell lines derived from a mouse transgenic for SV40 large T antigen. SV40 large T antigen expression is plotted as a percent of the highest expressing cell line (*MPCT1*) (*solid bars*). $n = 3$. + = $P < 0.01$ vs. MPCT1. Cells treated for 96 h with EGF (10^{-8} M) and TGFβ1 (10^{-10} M). The ratio of protein/DNA is plotted as a percent of control values (cells treated with vehicles only) (*open bars*). $n = 18$. * = $P < 0.01$ vs. untreated cell line. The slope of the regression between SV40 large T antigen expression and the protein/DNA ratio was significantly different from zero ($P < 0.05$). MMCD1, mouse medullary collecting duct cell line *1*; MMCD2, mouse medullary collecting duct cell line *2*; MPST1, mouse proximal straight tubule cell line *1*; MPCT1, mouse proximal convoluted tubule cell line *1*.



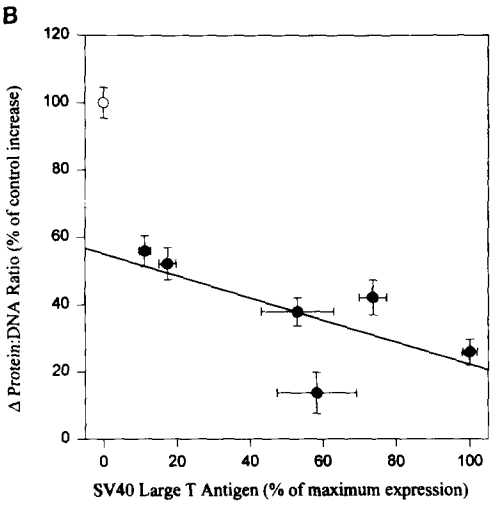


Figure 12. Correlation between EGF + TGFβ1-induced hypertrophy and the magnitude of SV40 large T antigen expression of NRK-52E clonal lines stably expressing the SV40 large T antigen gene. (*A*) Western blot showing SV40 large T antigen expression in 10 clones (lanes *1–10*). Lane *11* is a cell lysate from control infected cells (vector plus neomycin resistance gene only). (*B*) EGF + TGFβ1-induced hypertrophy as a function of the magnitude of SV40 large T antigen expression in 6 of the 10 clones. Cells were treated as in Fig. 11. SV40 large T antigen expression is plotted on the x-axis; the increase in protein/DNA in cells infected with pZIP-NeoSV(X)I, as a percent of that seen in cells infected with pZIP-Neo(X)I (*open circle*), is plotted on the y-axis. The slope of the regression between the protein/DNA ratio and SV40 large T antigen expression was significantly different from zero ($P < 0.01$). $n = 12$ per group for the protein/DNA measurements; $n = 3$ for the measurement of SV40 large T antigen expression.

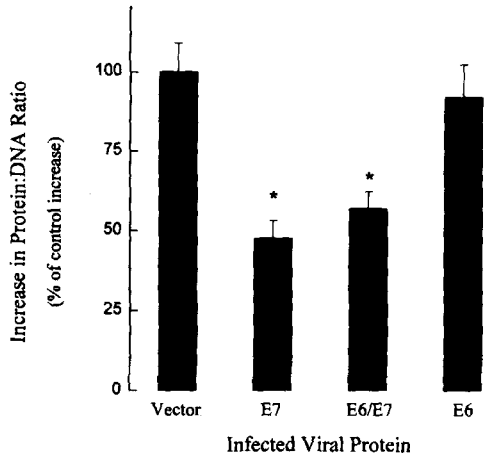


Figure 13. Effect of EGF + TGFβ1 on the ratio of protein/DNA in NRK-52E cells infected with HPV16. Cells were infected with HPV16 E6 (*E6*), HPV16 E7 (*E7*), or HPV16 E6 and E7 (*E6/E7*) as described in the Materials and Methods section. Cells were treated as in Fig. 11. The ratio of protein/DNA is plotted on the y-axis as a percent of EGF + TGFβ1-induced hypertrophy in control infected cells. $n = 12$ per group. * = $P < 0.01$ vs. vector.

proteins, but would prevent DNA synthesis and subsequent cell division. To address whether this is a feasible model for renal tubular hypertrophy, we treated primary cultures of proximal tubule cells with EGF, a growth factor known to induce proximal tubule cells to enter the growth cycle, and TGFβ1, a cytokine known to inhibit growth in many cells by G_1/S blockade. EGF alone increased DNA synthesis and DNA content/well and stimulated protein synthesis, without affecting the ratio of cell protein/DNA. TGFβ1 alone inhibited DNA synthesis, reduced DNA content/well, and had no effect on protein synthesis. The lack of an effect on basal protein synthesis coupled with a decrease in DNA synthesis is responsible for the small increase in the ratio of protein/DNA that was observed. However, the combination of TGFβ1 and EGF resulted in the development of hypertrophy.

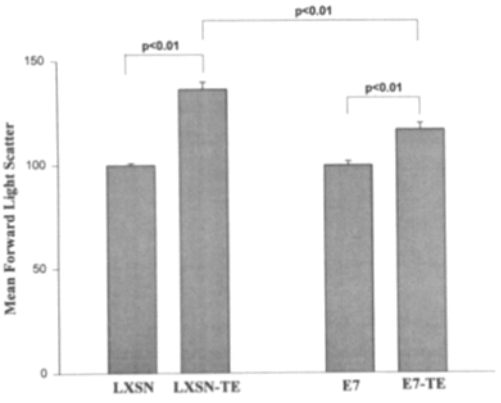


Figure 14. Effect of EGF + TGFβ1 on mean forward light scatter in HPV16 E7 infected cells. Cells were rendered quiescent for 48 h and then treated with vehicle (*LXSN* and *E7*) or the combination of EGF + TGFβ1 (*LSXN-TE* and *E7-TE*) for 48 h before study. For each group mean forward light scatter is plotted on the y-axis as a percent of control. LXSN, infected with vector alone; E7, infected with HPV16 E7.

The present results are consistent with the role of TGFβ in other models of hypertrophy. In primary cultures of vascular smooth muscle, TGFβ is associated with significant hypertrophy in the presence of serum, but with only minimal hypertrophy in the absence of serum (42). In primary cultures of rabbit proximal tubule cells, TGFβ converted insulin plus hydrocortisone-mediated hyperplasia to hypertrophy (12). Autocrine effects of secreted TGFβ appear to mediate angiotensin II-induced hypertrophy in mouse cortical tubule (MCT, a renal cell line) and vascular smooth muscle cells, and glucose-induced hypertrophy in MCT and mesangial cells, in that TGFβ neutralizing antibodies convert the hypertrophy to hyperplasia (17, 46, 52). Thus, TGFβ-mediated inhibition of hyperplasia plays an important role in the development of hypertrophy in many experimental models.

Our results suggest that the conversion of EGF-induced hyperplasia to hypertrophy by TGFβ is mediated through regulation of the pRB family of proteins. TGFβ inhibited EGF-induced pRB hyperphosphorylation, thus maintaining pRB in its active form. To address the role of pRB in the development of hypertrophy, we examined the effect of inhibiting pRB. In cells expressing SV40 large T antigen, hypertrophy was inhibited, and the degree of hypertrophy correlated inversely with large T antigen expression. Because SV40 large T antigen binds a number of nuclear proteins, including pRB, pRB-related proteins, and p53, we used a second approach. In cells infected with HPV16 E7, which inactivates pRB and pRB-related proteins, hypertrophy was inhibited. Conversely in cells infected with HPV16 E6, which degrades p53, hypertrophy was unaffected. It also remains possible that another protein that can be inactivated by both SV40 large T antigen and HPV16 E7, but not HPV16 E6, is part of the signaling pathway in TGFβ-dependent hypertrophy.

These studies agree with the important role that maintenance of an active member of the pRB family plays in the antiproliferative effects of TGFβ. In cells in which TGFβ is growth suppressive this effect is mediated by G_1/S blockade, is accompanied by inhibition of pRB phosphorylation, and is inhibited by SV40 large T antigen (15, 23, 32, 44).

An unresolved major question is the mechanism by which pRB phosphorylation is inhibited by TGFβ. pRB is phosphorylated by G_1 cyclin-dependent kinases, likely either cdk4/cyclin D or cdk2/cyclin E kinases. The activity of these kinases can be regulated by the abundance of either the cdk or cyclin molecule, the ability of the respective cdk and cyclin to form a stable complex, the rate of cdk phosphorylation, and/or the presence of cyclin-dependent kinase inhibitors, particularly p21, $p27^{KIP1}$, and $p15^{INK4B}$. In TGFβ-induced arrest of hyperplasia, TGFβ prevents the stable assembly of and/or activation of cdk2/cyclin E (28). In addition, TGFβ reduces cyclins E and A mRNA and protein abundance, inhibits cdk2 and cdk4 synthesis, and increases $p15^{INK4B}$ abundance, all of which can secondarily inhibit cdk2/cyclin E activity (10, 16, 18, 43). The role of these kinases in hypertrophy remains to be defined.

In summary, TGFβ converts EGF-induced hyperplasia into hypertrophy by inhibiting progression from G_1 to S phase. Inhibition of pRB phosphorylation and maintenance

of an active member of the pRB family appears to play a significant role in this effect. Blockade at the G_1/S transition in this model is TGFβ-dependent, but may be mediated in in vivo hypertrophy by other cytokines, by contact inhibition or by terminal differentiation, as in cardiac myocytes. The pRB family of proteins may play an important role in some or all of these processes. Evidence that cells enter the growth cycle in in vivo models of hypertrophy has been provided by demonstrated increases in immediate early gene expression in cardiac and renal hypertrophy (25, 29, 38, 47, 50). However, this effect can be somewhat nonspecific, and results in renal hypertrophy have not been consistent (40, 41). The present studies raise the possibility that the sclerosis and disease progression seen in hypertrophy may be secondary to signals generated by persistently activated pRB or a related protein, possibly in combination with other G_1 signaling events.

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