Transforming growth factor-β1 up-regulates p15, p21 and p27 and blocks cell cycling in G1 in human prostate epithelium

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Abstract

Transforming growth factor-β1 (TGFβ1) is inhibitory to most epithelia, but its role in the control of proliferation of prostatic epithelium is unclear. In some cells, TGFβ1 inhibition is achieved by up-regulation of cyclin-dependent kinase (cdk) inhibitors including p15, p21 and p27.

Our aims were to determine whether the effects of TGFβ1 on human prostatic epithelial cell cycle kinetics were mediated by alterations in the levels of the cdk inhibitors p15, p16, p21 and p27 and hypo-phosphorylated retinoblastoma protein (Rb).

Human prostatic epithelial cells in primary culture were grown in the presence of TGFβ1 (0–10 ng/ml) for up to 4 days and proliferation assessed using a [^{3}H]thymidine uptake assay. Levels of p15, p16, p21 and p27 were measured at both mRNA and protein level by means of a reverse transcriptase PCR-based assay and Western analysis. Rb and cdk2 levels were measured.

Exogenous TGFβ1 (0–5 ng/ml) inhibited proliferation. This was associated with blocking of the cell cycle at G1, and up to 4-fold increases in p15, p21 and p27 mRNA levels, but no change was observed in p16 mRNA levels; these changes were not blocked by cycloheximide. Increased levels of p15, p21 and p27 protein were also accompanied by increased levels of hypo-phosphorylated Rb and decreased cdk2 kinase activity.

TGFβ1 has mainly inhibitory effects on benign human prostatic epithelium, which are caused by up-regulation of cdk inhibitors, hypo-phosphorylation of Rb and delaying of the cell cycle in G1.

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Introduction

Benign enlargement of the prostate (benign prostatic hyperplasia (BPH)) is very common, being found in 40% of men aged greater than 60 years and necessitating an operation in about 15%. Complex endocrine, autocrine and paracrine interactions between prostatic epithelium and stroma may be responsible for the formation of BPH. Prostate cancer is the second most common malignancy in men, and in its advanced stages is unresponsive to androgen control, which may be mediated by alterations in peptide growth factor function.

Transforming growth factor-β1 (TGFβ1) is widely distributed. In general, TGFβ1 stimulates mesenchyme and inhibits epithelial growth (Moses *et al.* 1990). TGFβ1 and TGFβ2 have been identified in human prostate and implicated in prostatic disease (Knabbe *et al.* 1993, Truong *et al.* 1993). Addition of TGFβ1 to cultured prostatic epithelial and stromal cells has been reported to inhibit proliferation in both cell types (Sutkowski *et al.* 1992). However, low levels of TGFβ1 have been shown to result in small increases (1·5-fold) in cell proliferation in both stroma and epithelium (Collins *et al.* 1996).

Cell cycle progression is governed by a series of cyclin-dependent kinases (cdks) whose function is dependent on their phosphorylation status and association with specific cyclins (Sherr 1994). Positive regulatory factors include growth factors, transcription factors, cyclins and cdks. Negative regulatory forces are provided by tumour suppressor proteins, notably those encoded by the tumour suppressor genes, p53 and Rb (retinoblastoma) and the cdk inhibitors (CDKIs). The D family of cyclins complex with cdk4 and cdk6, while the principal partner of cyclin E is cdk2. The Rb protein binds to and sequesters specific proteins necessary for cell cycle progression, thereby preventing cell cycle progression. During G1 phase, Rb protein is phosphorylated by cyclin D/cdk4 and cyclin D/cdk6 complexes. Phosphorylation of Rb inactivates it and allows the release of E2F, thereby allowing entry of cells into S phase (Weinberg 1995).

CDKIs control the phosphorylation status of Rb protein (Clurman & Roberts 1995). The Kip/cip members, which include p21, p27 and p57, are capable of binding to

and inhibiting most cyclin/cdk complexes. Their expression is dependent on up-stream events which are activated by physiological signals such as DNA damage, serum deprivation or contact inhibition. Up-regulation of p21 is thought to be the means by which p53 places dividing cells in G1 phase arrest in response to DNA damage (Macleod *et al.* 1995). In contrast the INK4 family, which includes p15, p16, p18 and p19, bind to and inactivate D-type cyclins. Studies of p16 and p15 (INK4a and INK4b) have shown that they can function as tumour suppressors (Cairns *et al.* 1994, Quelle *et al.* 1995).

In keratinocytes, p15 is up-regulated 30-fold by 2 ng/ml TGFβ1 (Hannon & Beach 1994), though this study failed to demonstrate changes in p16 or p27. In gastric cancer cell lines, TGFβ1 up-regulates p21 about 3-fold (Akagi *et al.* 1996), but produces only slight changes in expression of p27. Although p27 was originally identified in cells treated with TGFβ1 (Polyak *et al.* 1994), recent studies have failed to demonstrate significant changes in mRNA levels, but protein levels may be altered by sequestration to cyclin D. Decrease in cdk4 following application of TGFβ1 may release previously bound p27 protein.

The purpose of this study was to investigate how high levels of TGFβ1 result in inhibition of human prostate cells in primary culture. We believe that investigation of CDKI regulation by TGFβ1 in the prostate has not been performed previously. The results show the complexity of growth control in the human prostate epithelium and that p15, p21 and p27 are the main mediators of inhibition resulting in an accumulation of cells in G1.

Materials and Methods

Preparation of primary cultures of human prostatic epithelial and stromal cells

Prostatic tissue was obtained from men undergoing open prostatectomy and transurethral prostatic resection for bladder outflow obstruction. Specimens were dissected into 1–2 mm^3 pieces, but representative chips were retained to confirm the histological diagnosis of BPH. Tissue was then incubated at 37 °C for 20 h with collagenase type I (2000 IU/ml) (Lorne Laboratories, Reading, UK) in RPMI-1640 (Life Technologies Ltd, Paisley, Scotland) containing 5% foetal calf serum (Life Technologies Ltd). The digest was centrifuged at 800 *g* for 10 min and supernatant discarded. The cell pellet was re-suspended in 10 ml Dulbecco 'A' PBS (Oxoid Ltd, Basingstoke, Hampshire, UK) and centrifuged at 800 *g* for 10 min. This latter step was repeated. The cell pellet was then re-suspended in RPMI-1640 and centrifuged twice at 400 *g* for 1 min. Epithelial cells were collected as a pellet following each centrifugation. Stromal cells remained in the supernatant. Epithelium was grown in primary growth medium in 75 cm^2 tissue culture flasks

(Becton Dickinson, Oxford, UK) at 37 °C in 5% CO_2. This method has been shown to produce primary cultures that are at least 95% pure (Collins *et al.* 1994).

Cell culture media

Epithelial cells were cultured in complete WAJC-404 serum-free medium (WAJC-404 basal medium; Life Technologies Ltd) supplemented with insulin (2·5 mg/ml; Sigma Chemical Co. Ltd, Poole, Dorset, UK), dexamethasone (1 µM; Sigma), epidermal growth factor (EGF) (10 ng/ml; Sigma), bovine pituitary extract (25 mg/ml; Sigma), cholera toxin (10 ng/ml; Sigma), heparin (25 ng/ml; Sigma), penicillin (100 units/ml; Life Technologies Ltd) and streptomycin (100 mg/ml; Life Technologies Ltd). WAJC-404 medium has a low Ca^{2+} concentration that prohibits stromal cell growth. Basal epithelial growth medium contained all the above supplements apart from EGF and bovine pituitary extract and included low levels of fibroblast growth factor–2 (FGF2) (10^{-9} M).

Mitogenesis assays and addition of TGFβ1

Sub-confluent primary monolayer cultures of epithelial cells were washed with PBS and then detached using 0·25% trypsin, 0·02% EDTA (Life Technologies Ltd). Cells were plated at a density of 1×10^4 cells/well in 96-well plates in primary epithelial growth medium. After 24 h, primary growth medium was removed and replaced by basal medium. Cells were then cultured for 4 days, after which 1 µCi [6-^{3}H]thymidine (5·0 Ci/mmol, Amersham International, Amersham, Bucks, UK) in basal epithelial growth medium was added to each well. Cells were harvested 12 h later. Medium was aspirated from each well and 50 µl trypsin (2·5%) was added. After 20 min, 10% trichloroacetic acid (4 °C) was added and cells incubated at 4 °C for 2 h. Plates were harvested on filter mats (Tomtec cell harvester, Wallac, Milton Keynes, Oxfordshire, UK), dried at 60 °C for 30 min and counted following the addition of scintillation fluid (BetaPlate Scint; Wallac, Milton-Keynes, UK) using a 1450 Microbeta Plus scintillation counter (Wallac). Recombinant TGFβ1 protein (R&D Systems, Abingdon, UK) was added to the culture medium at concentrations from 0·001 to 100 ng/ml. For some experiments investigating the duration of exposure to TGFβ1 on cell proliferation, three low concentrations were used (0·03, 0·01 and 0·003 ng/ml). In order to determine the effects of TGFβ1 on expression of cdks, cells were exposed to 0, 0·03, 0·1, 0·3, 1 and 5 ng/ml for 96 h.

To test the effect of TGFβ1 on proliferation, TGFβ1 neutralising antibody (10–30 µg/ml; R&D Systems) or TGFβ2 neutralising antibody (10–30 µg/ml; R&D Systems) was added to the epithelial compartment and [^{3}H]thymidine incorporation was measured after 4 days as described. Both anti-TGFβ1 and anti-TGFβ2 antibodies

are highly specific and do not cross-react with each other. The effect of neutralising antibody was carried out in the absence of low levels of FGF2 as we wished to simply determine whether TGFβ1 was present.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cell cultures were lysed directly into guanidinium thiocyanate and total RNA was extracted according to Chomczynski & Sacchi (1987). First strand cDNA was made from 5 µg RNA using AMV Reverse Transcriptase (Krammel Biotech, Cramlington, UK). PCR was performed with 1/25th of the cDNA using Taq polymerase (Perkin Elmer Cetus, Cheshire, UK) and standard PCR conditions. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 25 cycles of PCR were performed using the following oligonucleotides: 5′ AGT CAA CGG ATT TGG TCG TA 3′ and 5′ AAA TGA GCC CCA GCC TTC T 3′ to produce a 315 bp product. For p15, p16, p21 and p27 the cDNA fragments were amplified using 35 cycles of PCR with the following oligonucleotide pairs: p15 5′ GGC GCG CGA TCC AGG TCA 3′, 5′ GAA CCT GGC GTC AGT CCC 3′ (285 bp product); p16 5′ CGG AAG GTC CCT CAG ACA TC 3′, 5′ TCA TGA AGT CGA CAG CTT CCG 3′ (385 bp product); p21 5′ CTC AGA GGA GGC GCC ATG 3′, 5′ GGG CGG ATT AGG GCT TCC 3′ (517 bp product); p27 5′ AGG ATG TCA GCG GGA GCC G G 3′, 5′ CTT CTT GGG CGT CTG CTC CA 3′ (250 bp product). Oligonucleotides were designed to be highly specific and to cross intron–exon boundaries. A range of PCR cycles were evaluated (GAPDH, 20–25 cycles; p15, p16, p21, p27, 30–35 cycles) to determine the linear range necessary for cDNA amplification. To enable quantification of cDNA products the PCR reactions were modified to include 20 µM dNTPs and 0·25 µCi [α-^{32}P]dATP (ICN, Thame, Oxon, UK). PCR products were resolved on 4% 90 mM Tris, 90 mM boric acid, 2 mM EDTA–polyacrylamide gels. The gels were dried and exposed to autoradiography film or a phosphor screen (Molecular Dynamics, Chesham, Bucks, UK) to allow accurate quantification of products. Results were expressed as the ratio of CDKI: GAPDH. A range of PCR cycles were employed for each cDNA to verify amplification was in the linear range of the PCR process.

Addition of cycloheximide

Cells were exposed to 10 mg/ml cycloheximide (Sigma) for 1 h and 5 ng/ml TGFβ1 subsequently added for 0, 12, 24, 48 and 96 h.

Western blotting

Following exposure to TGFβ1, cultured cells were detached with trypsin and immediately re-suspended in SDS loading buffer and boiled for 10 min. Electrophoresis was performed using 12% SDS-polyacrylamide gels (Bio-Rad Minigel System, Bio-Rad Laboratories, Hemel Hempstead, Herts, UK) and proteins were transferred to nitrocellulose membranes (Amersham International). Membranes were blocked in 20 mM Tris-HCl pH 7·5, 500 mM NaCl containing 5% dried milk and 0·1% Tween-20 and probed with rabbit anti-p15 polyclonal antibodies (Santa Cruz), rabbit anti-p16 polyclonal antibody (Novocastra, Newcastle, Tyne and Wear, UK; Santa Cruz, CA, USA), mouse anti-p21 monoclonal antibody (Santa Cruz), rabbit anti-p27 polyclonal antibody (Santa Cruz), mouse anti-Rb monoclonal antibody (PharMingen, San Diego, CA, USA) and mouse anti-β-tubulin monoclonal antibody (Sigma) for 1 h. Following a second incubation with peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Dako, Ely, Cambridgeshire, UK) proteins were visualised using an enhanced chemiluminescent detection system (Amersham International).

Flow cytometry

Flow cytometry was used to analyse the effects of TGFβ1 on the cell cycle and to quantify immunofluorescence for the cellular proliferation marker Ki-67. All manipulations were performed on ice to minimise damage to cells. Cells were detached from Petri dishes using PBS, 0·02% EDTA and washed using an automatic cell washer (Diacent 2000, Rosslab, Macclesfield, UK). For studies involving immunofluorescence staining, the cells were fixed by re-suspension in 200 µl cold methanol and incubation at − 70 °C, then washed by centrifugation. Pellets were re-suspended in isoton (Coulter, Luton, Bedfordshire, UK) and saponin was added to 1% to permeabilise the cells. Cells were incubated in the presence of fluorescein isothiocyanate (FITC)-conjugated mouse anti-Ki-67 monoclonal antibody (Immunotech, Marseille, France) on ice for 20 min at a 1:20 dilution. Unbound antibody was removed by centrifugal washing. Control cell pellets were re-suspended in FITC-conjugated goat anti-mouse antibody (Becton Dickinson) at an equivalent concentration and treated in an identical fashion. Cells were washed by centrifugation and re-suspended in isoton containing propidium iodide (0·25 mg/ml) and RNaseA (1 mg/ml) to stain DNA. Samples were analysed on a bench-top FAC-Scan flow cytometer (Becton Dickinson); cellular fluorescence was measured after argon laser excitation at 488 nm. Data were analysed using the LYSIS II software package (Becton Dickinson) and statistics calculated using Microsoft Excel.

Immunoprecipitation and histone H1 kinase assays

Cells were washed with ice-cold PBS, pelleted and lysed in immunoprecipitation buffer (50 mM Tris–HCl pH 7·5, 150 mM NaCl, 0·5% NaPO$_4$, 50 mM NaF, 0·2 mM
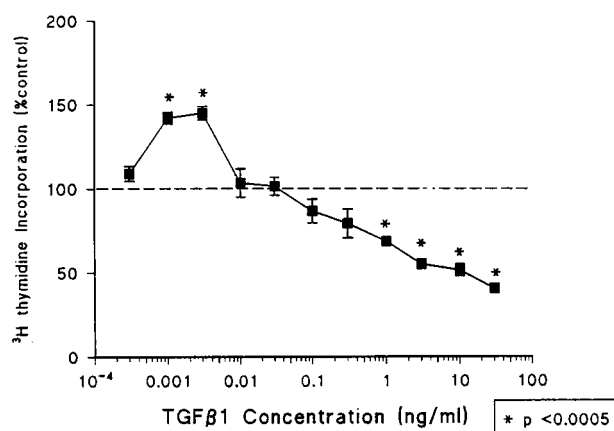
Figure 1 Effect of exogenous TGFβ1 on epithelial proliferation. Exogenous TGFβ1 (0·0003–0·3 ng/ml) was added directly in basal epithelial medium with 10^{-9} M FGF2 to epithelial cells. All measurements were performed in quadruplicate. [^{3}H]Thymidine incorporation was measured after 4 days. Results are expressed as a percentage of the untreated control (epithelium in basal medium with 10^{-9} M FGF2). Statistical analysis compares each point against untreated control.

Figure 2 Effect of TGFβ1, TGFβ2 and non-specific neutralising antibodies on epithelial proliferation. Neutralising antibodies to TGFβ1, TGFβ2 and a non-specific chicken antibody were added to epithelial cells in basal epithelial medium. All measurements were performed in quadruplicate. [^{3}H]Thymidine incorporation was measured after 4 days. Results are expressed as a percentage of untreated control (epithelium in basal medium). Statistical analysis compares each point against untreated control. NAb=neutralising antibody.

NaVaO$_4$, 1 mM polymethylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 25 µg/ml aprotinin, 25 µg/ml leupeptin, 25 µg/ml pepstatin). Lysates were cleared by centrifugation at 33 000 *g* for 20 min. Two hundred micrograms total protein were incubated for 2 h with anti–cdk2 antibody (Santa Cruz) which had previously been incubated with protein A–agarose beads (Pharmacia, Milton Keynes, Bucks, UK). Cdk2-associated kinase activity was assayed in anti–cdk2 immune complexes. The beads were washed three times in immunoprecipitation buffer and three times in kinase reaction buffer (50 mM Hepes pH 7·5, 10 mM MgCl$_2$, 5 mM MnCl$_2$, 1 mM DTT, 50 mM NaF, 0·2 mM NaVaO$_4$, 1 mM PMSF, 25 µg/ml aprotinin, 25 µg/ml leupeptin, 25 µg/ml pepstatin). Kinase reactions were performed at 37 °C for 30 min in 20 µl kinase buffer containing 0·1 µCi [γ-^{32}P]ATP (ICN, UK) and 2 µg histone H1 substrate (Boehringer-Mannheim, Lewes, East Sussex, UK). The reactions were terminated with 5 × SDS sample buffer. Following boiling for 5 min and centrifugation for 2 min at 5000 *g* the supernatants were resolved on 12% denaturing polyacrylamide gels. Radioactivity was detected by autoradiography or phosphor imager (Molecular Dynamics, UK).

Statistical analysis

Statistical significance was assessed using ANOVA (InStat GraphPad Software, San Diego, CA, USA). Two-tailed probabilities of less than 0·05 were considered as significant.
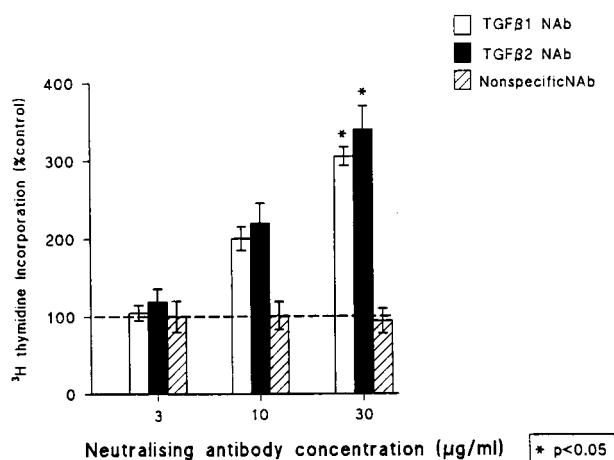
Results

Effect of TGFβ1 on epithelial proliferation

The effect of varying concentrations of TGFβ1 on epithelial proliferation from a representative primary culture is shown in Fig. 1. In the presence of low levels of FGF2 (10^{-9} M), low TGFβ1 concentrations (0·001–0·003 ng/ml) caused stimulation of proliferation by 50% compared with control levels (control=340 ± 35 c.p.m.). A dose-dependent decrease in proliferation was observed with TGFβ1 concentrations greater than 0·01 ng/ml. This biphasic effect on DNA synthesis was observed with each epithelial culture tested, but only in the presence of FGF2 (R L Byrne and A T Collins, unpublished results).

Blocking TGFβ1 action by co-culture with antibody (10 and 30 µg/ml) in the absence of added FGF2 (10^{-9} M) resulted in down-regulation of TGFβ1 inhibition and therefore increased proliferation (Fig. 2).

Effect of TGFβ1 on mRNA levels of CDKIs

Increasing doses of TGFβ1 (0–5 ng/ml) were applied to epithelial cells for a period of 4 days and mRNA transcripts for p15, p16, p21 and p27 were measured by RT-PCR (Fig. 3, upper panel). The expression of CDKIs was quantified relative to the expression of the housekeeping gene, GAPDH (Fig. 3, lower panel). The level of p15 mRNA rose with increasing doses of TGFβ1 for the 4 day exposure. Following an exposure to 5 ng/ml TGFβ1 the level of p15 mRNA increased by
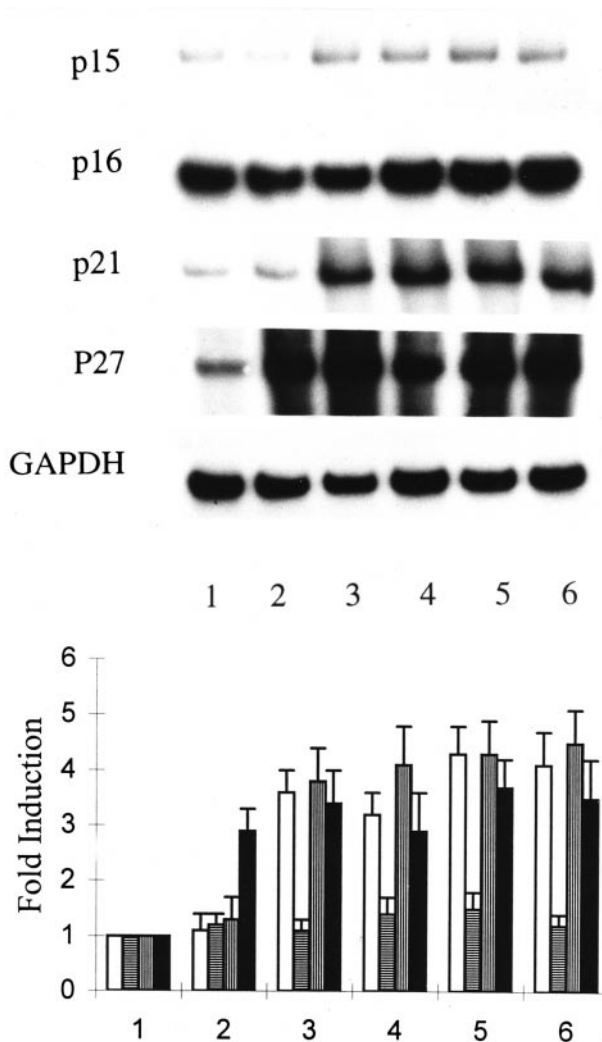
Figure 4 Effect of TGFβ1 on p15, p21, p27 and β-tubulin protein expression. Primary prostatic epithelial cells were exposed to basal medium in the absence (lane 1) or presence (lanes 2–6, 0·03, 0·1, 0·3, 1, 5 ng/ml respectively) of TGFβ1 for 4 days. Equivalent amounts of cellular lysates were subjected to Western analysis to determine the expression of p15, p21, p27 and β-tubulin protein.

Figure 3 Effect of TGFβ1 on p15, p16, p21, p27 and GAPDH mRNA expression. Primary prostatic epithelial cells were exposed to basal medium in the absence (lane 1) or presence (lanes 2–6, 0·03, 0·1, 0·3, 1, 5 ng/ml respectively) of TGFβ1 for 4 days. Upper panel: mRNA was detected by RT-PCR following PAGE. Lower panel: mRNA expression was quantified relative to the expression of GAPDH. The results represent the mean ± S.D. of at least three independent experiments. Clear box, p15; horizontal bands, p16; vertical bands, p21; black, p27.

approximately 4-fold relative to the level of GAPDH. No detected change was noted for p16 mRNA with a range of TGFβ1 exposures. Both p21 and p27 mRNA levels increased with higher concentrations of TGFβ1. For a dose of 5 ng/ml TGFβ1 the levels of p21 and p27 rose by 4·5- and 3·5-fold respectively. All reported differences were highly significant ($P<0·01$).

Effects of TGFβ1 on protein levels of CDKIs

Epithelial cells were exposed to increasing concentrations of TGFβ1 (0–5 ng/ml) for a period of 4 days and the levels
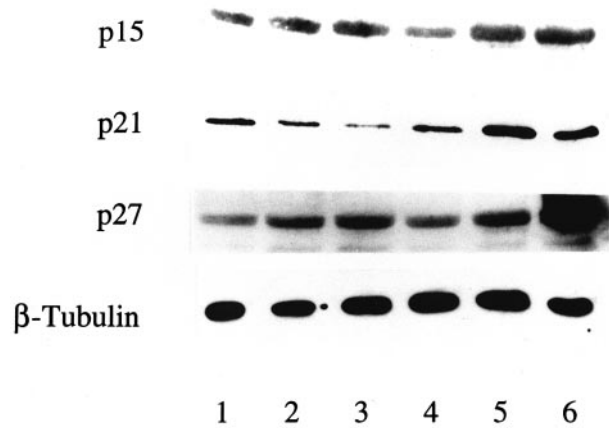
of CDKI proteins were measured by Western analysis performed on cell extracts (Fig. 4). Blots were subsequently re-probed with β-tubulin to confirm equal loading of cellular protein. The level of p15, p21 and p27 protein increased with the dose of TGFβ1. The greatest increase relative to control levels was noted with 5 ng/ml TGFβ1 for p15, p21 and p27. Densitometric analysis indicated increases in expression of between 4- and 5-fold with 5 ng/ml TGFβ1 for p15, p21 and p27 with independent cultured epithelial cells (data not shown). No detected expression of p16 protein was observed using two different anti-p16 antibodies, suggesting low level expression of this protein in primary cultured prostatic epithelial cells.

Temporal expression of CDKIs following TGFβ1 exposures

A single growth inhibitory dose of 5 ng/ml TGFβ1 was given to cells for increasing periods of time (12, 24, 48 and 96 h) and the level of mRNA expression of CDKIs was measured by RT-PCR (Fig. 5, upper panel). The change in CDKI mRNA expression was assessed relative to the expression of GAPDH (Fig. 5, lower panel). A rapid induction in the level of p15 mRNA was observed. Following a 12 h exposure to 5 ng/ml TGFβ1 the level of p15 mRNA increased approximately 4·5-fold relative to the control level. The accumulation of p15 mRNA increased with time to 48 h (6·5-fold). A similar rapid induction in the level of both p21 and p27 was noted. At 12 h post-TGFβ1 exposure the level of p21 and p27 increased 1·2- and 3·4-fold respectively. The levels of these mRNAs showed a further increase in expression up
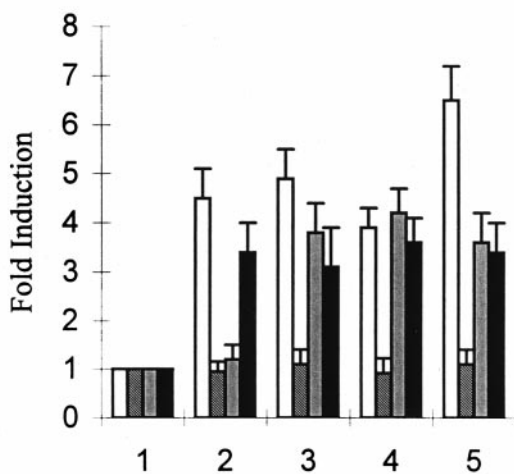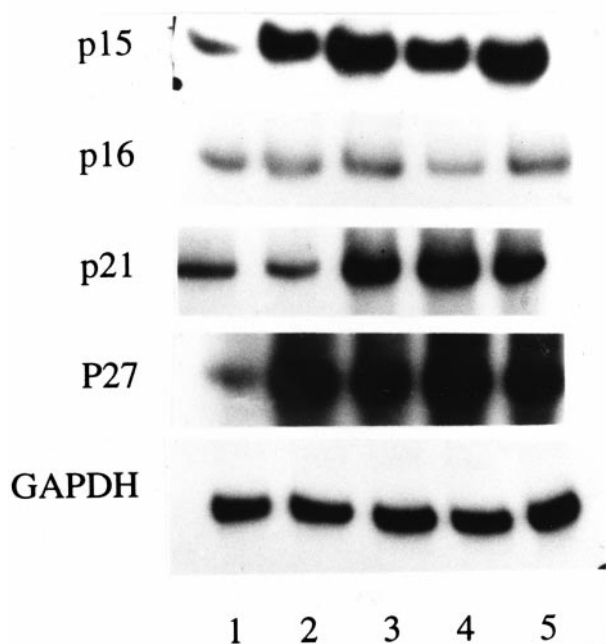
Figure 5 Temporal effect of TGFβ1 on p15, p16, p21, p27 and GAPDH mRNA expression. Primary prostatic epithelial cells were exposed to basal medium in the absence (lane 1) or presence of 5 ng/ml TGFβ1 for periods of 12, 24, 48 and 96 h (lanes 2–5 respectively). Upper panel: mRNA was detected by RT-PCR following PAGE. Lower panel: mRNA expression was quantified relative to the expression of GAPDH. The results represent the mean ± S.D. of at least three independent experiments. Clear box, p15; horizontal bands, p16; vertical bands, p21; black, p27.

to 96 h exposure to TGFβ1 (3·6- and 3·4-fold respectively). A single dose of TGFβ1 given to the cultured epithelial cells produced no change in p16 mRNA level, which was found at very low levels in the cell culture system described here. All reported differences were highly significant ($P < 0.01$).
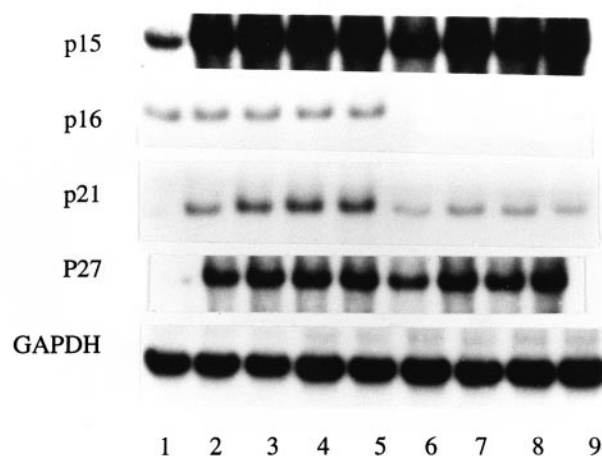
Figure 6 Effect of cycloheximide on TGFβ1-induced alterations in p15, p16, p21, p27 and GAPDH mRNA expression. Primary prostatic epithelial cells were exposed to basal medium in the absence (lane 1) or presence of 5 ng/ml TGFβ1 for periods of 12, 24, 48 and 96 h (lanes 2–5 respectively), or the presence of TGFβ1 and cycloheximide for periods of 12, 24, 48 and 96 h (lanes 6–9 respectively). mRNA was detected by RT-PCR following PAGE.

Effects of cycloheximide on TGFβ1-induced changes in expression of CDKIs

The effect of addition of the protein synthesis inhibitor cycloheximide, prior to exposure to 5 ng/ml TGFβ1 for periods up to 96 h, on the level of CDKI mRNAs was investigated by RT-PCR (Fig. 6). Changes in CDKI mRNAs were measured relative to the level of GAPDH. Cycloheximide failed to prevent the rapid induction of p15 and p21 mRNAs observed following TGFβ1 administration for 12 h. The level of p15 rose approximately 5-fold after a 24 h exposure to TGFβ1, and between 48 and 96 h the level of p15 remained at about 4-fold above the control level. Cycloheximide exposure also failed to prevent the accumulation of p21 mRNA over the 96 h period. The p27 mRNA level showed a steady increase in expression in response to TGFβ1 over the 96 h period. The level of p27 mRNA rose to approximately 3·5-fold above the control level at 96 h. In response to combined treatment with TGFβ1 and cycloheximide the level of p16 mRNA was seen to decrease rapidly by approximately 4-fold at 12 h and this low level persisted up to 96 h.

Effect of TGFβ1 on cell cycle kinetics of epithelial cells

Epithelial cells were exposed to increasing concentrations of TGFβ1 (0–5 ng/ml) for a period of 4 days, removed using PBS/EDTA and analysed for distribution throughout the cell cycle (Fig. 7). These results were confirmed by measurement of cycling cells only (Ki67-positive) using
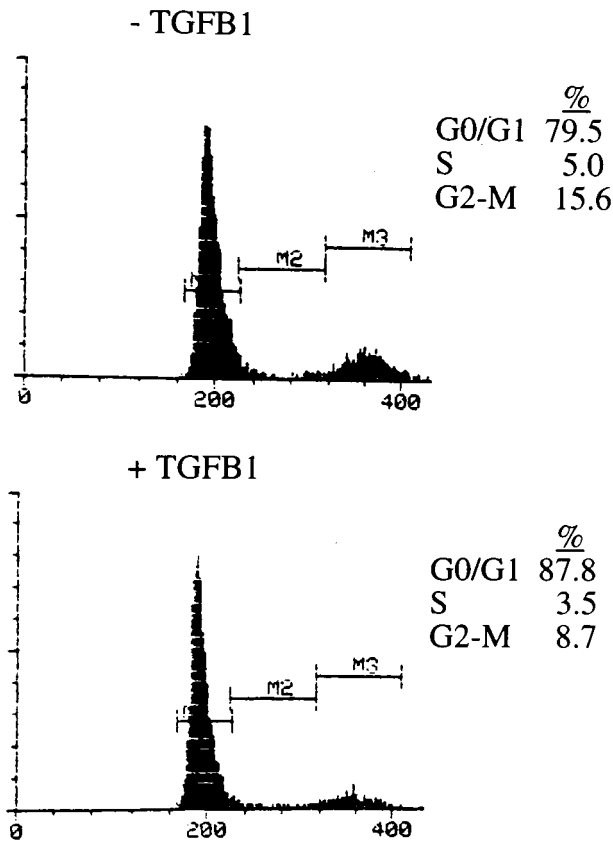
Figure 7 The effects of TGFβ1 on cell cycle kinetics of primary prostatic epithelial cells. Epithelial cells were exposed to basal medium in the absence or presence of 5 ng/ml TGFβ1 for 4 days. Cells were harvested and stained with propidium iodide and analysed using flow cytometry. The distribution of epithelial cells in the G0–G1 (M1), S (M2) and G2–M (M3) phases of the cell cycle was calculated using LYSIS II software (Becton Dickinson).

flow cytometry. In the absence of TGFβ1, there were 84, 5 and 11% of cells in G1, S and G2–M phases respectively. Addition of 5 ng/ml TGFβ1 changed the distribution of cycling cells so that 92, 3 and 5% of cells were in G1, S and G2–M phases of the cell cycle respectively. Ki67 is expressed in all cells except G0 and the addition of TGFβ1

causes a shift of cells into the G1 phase of the cell cycle suggesting this is where inhibition is most acute. These data show that TGFβ1 administration is associated with an increased number of cells being placed in G0/G1 (Fig. 7) compared with S or G2/M.

Effect of TGFβ1 on Rb protein expression

Epithelial cells were exposed to 5 ng/ml TGFβ1 for periods up to 4 days and the levels of Rb protein were measured by Western analysis performed on approximately equal amounts of cell extracts (Fig. 8). Densitometric analysis was performed to determine the change in Rb following TGFβ1 exposure. The levels of both hyper-phosphorylated Rb (ppRb) and hypo–phosphorylated Rb protein (pRb) were observed to increase with time for TGFβ1 exposure. However, the pRb form was noted to increase relative to the ppRb.

Effect of TGFβ1 on cdk2-associated kinase activity

Epithelial cells were cultured in the absence or presence of 5 ng/ml TGFβ1 for 4 days and assayed for cdk2-associated kinase activity following immunoprecipitaion with anti-cdk2 antibody (Fig. 9). Cells cultured in the absence of TGFβ1 demonstrated cdk2 kinase activity as evidenced by the ability to phosphorylate the histone H1 substrate. However, cells cultured in the presence of TGFβ1 demonstrated no detected cdk2-associated kinase activity, since they were unable to phosphorylate histone H1.

Cdk2 immunoprecipitated protein extracts were also resolved following SDS PAGE and subjected to Western analysis using anti–cdk2 antibody. Figure 9 shows similar levels of cdk2 protein were immunoprecipitated for cells cultured in the presence or absence of TGFβ1.

Discussion

Previous authors have shown that TGFβ1 has a predominantly inhibitory effect on the proliferation of human prostatic stroma and epithelium. However, some authors
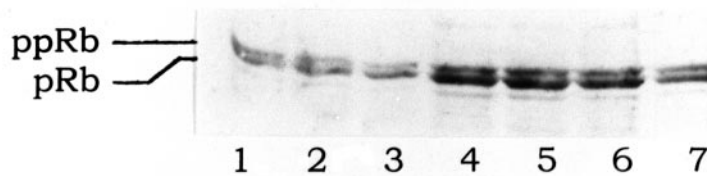

Figure 8 The effect of TGFβ1 on Rb protein expression. Primary prostatic epithelial cells were exposed to basal medium in the absence (lane 1) or presence of 5 ng/ml TGFβ1 for periods of 4, 8, 16, 24, 48 and 96 h (lanes 2–7 respectively). Equivalent amounts of cellular lysates were subjected to Western analysis to determine the expression of pRb and ppRb protein.
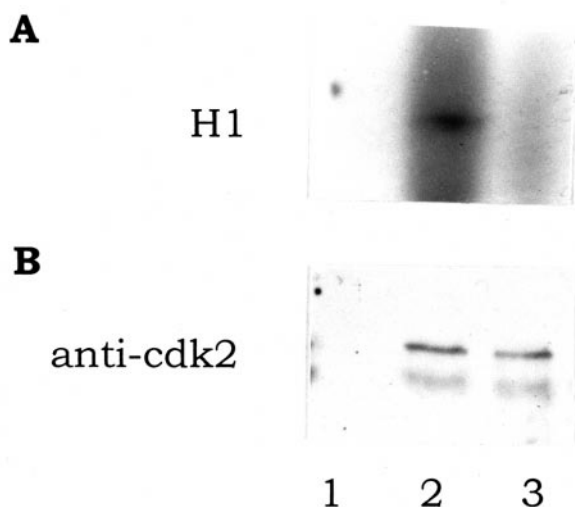
Figure 9 The effect of TGFβ1 on cdk2 kinase activity. Primary prostatic epithelial cells were exposed to basal medium in the absence or presence (lanes 2 and 3 respectively) of 5 ng/ml TGFβ1 for 96 h. A control reaction lacking cell extract was included (lane 1). Cells were immunoprecipitated with anti-cdk2 and assayed for histone H1 kinase activity (A) and also subjected to Western analysis (B).

have shown a biphasic effect on both cell types with low concentrations being associated with small increases in proliferation, which may be caused by secondary increases in mitogenic growth factors such as basic FGF (Story *et al.* 1993, Collins *et al.* 1996). Benign prostatic epithelial cells have been shown by immunohistochemistry to express mitogenic growth factors including FGF2 (Collins *et al.* 1996) and EGF (Shikata *et al.* 1984). It is possible that low concentrations of TGFβ1 in the context of a mixed epithelial/mesenchymal gland such as the prostate may cause the secondary release/synthesis of such growth factors, which may thereby mediate the biphasic effect of TGFβ1 in prostatic epithelium. Another reason for an apparent increase in proliferation may be the presence of mesenchymal cells which proliferate in response to TGFβ1 even in apparently pure preparations of human prostatic epithelium in primary culture.

Receptors for TGFβ1 have been identified in rat ventral prostate where they are negatively regulated by androgens and their up-regulation is involved in the mechanism of castration-induced prostatic cell death (Kyprianou & Isaacs 1988). Receptors for TGFβ1 have also been identified in the human prostate cancer cell lines DU145 and PC-3 (Wilding *et al.* 1989). TGFβ1 has a mainly inhibitory effect on human prostatic fibroblasts and epithelial cells in culture (Sutkowski *et al.* 1992, Story *et al.* 1993). Our studies have confirmed this mainly inhibitory effect, which was most marked in cultures which contained low levels of FGF2. The effect of neutralising

antibodies was carried out in the absence of any added mitogens and we observed release of inhibition caused by the presence of TGFβ1 secreted by the cells.

Immunohistochemical studies (Truong *et al.* 1993) have identified TGFβ1 in human prostate and significantly greater staining was found in cancer compared with benign (BPH) specimens. Wilding *et al.* (1989) found that the androgen-independent cell lines DU145 and PC-3 were inhibited by TGFβ1, but the androgen-sensitive LNCaP cell line showed no response. In addition, the androgen-independent cell lines were able to secrete TGFβ1 and contained a high-affinity receptor for TGFβ1. Thus, in androgen-independent cell lines, there exists an inhibitory autocrine loop for control of cellular proliferation by TGFβ1. Although increasing levels of TGFβ1 usually cause an initial inhibition of cell proliferation, further administration can lead to increased growth rates in rat prostate cancer cell lines (Shain *et al.* 1990). Thus, the effect of TGFβ1 is dependent on the cell type examined. Other functions of TGFβ1 that would support growth of tumour cells and may encourage metastasis include its association with extracellular matrix (Ignotz & Massagué 1986), its ability to inhibit T and B lymphocytes leading to defects in host immunity (Kehrl *et al.* 1986, Torre-Amione *et al.* 1990), and the ability to act as a potent angiogenic factor (Yang & Moses 1990). Understanding the function of TGFβ1 in both normal and malignant prostate is further complicated by its interaction with other growth factors which influence cell proliferation. Nevertheless, inhibition is the main effect of TGFβ1 in prostate stroma and epithelium.

TGFβ1 binds to serine/threonine kinase receptors (Type I and II), which form heterodimers in the presence of ligand. How this results in the downstream events of TGFβ1, including induction of some CDKIs, is unclear (Massagué *et al.* 1994). Some members of the TGFβ1 super-family, including bone morphogenetic proteins (BMPs), result in accumulation of Drosophila MADR1, a homologue of human MAD (Hoodless *et al.* 1996). Recent studies have shown that binding of TGFβ1 results in activation of Smad 2 and Smad 3, which associate with Smad 4 to move to the nucleus. Some SMAD proteins are the target of receptor tyrosine kinases such as the EGF receptor. Such SMAD phosphorylation mediated by the Erk family of MAP kinases inhibits the nuclear localisation of Smad 1 initiated by binding of BMPs (Kretzschmar *et al.* 1997) to TGFβ1 receptors. This demonstrates the complex interaction between growth factors.

Our study was mainly directed towards the negative regulatory functions of TGFβ1, which appear to be produced, at least in part, by up-regulation of CDKIs. Whilst p27 was initially identified in TGFβ1-stimulated cells, p27 mRNA levels are not usually induced and this may imply some post-translational alteration in protein binding with cyclins. Indeed culture of pituitary cells with 10^{-9} M TGFβ1 decreased p27 mRNA and protein levels

(Qian *et al.* 1996). Moreover, cells from mice lacking p27 still respond to TGFβ1 by inhibition (Nakayama *et al.* 1996). In our experiments the level of p27 mRNA showed an approximately 3·5-fold increase in response to a growth inhibitory dose of TGFβ1 with a corresponding 4-fold increase in the level of p27 protein. Our finding that TGFβ1 induced the expression of p15 is in keeping with previous data (Hannon & Beach 1994) demonstrating a significant up-regulation by 30-fold. It is not surprising that such massive increases in expression were not found in our experiments on primary human cells from the prostate, because many of these cells are not actively dividing and may be apoptotic. p21 is normally induced by increases in the levels of wild type p53 (Macleod *et al.* 1995) and in addition to being a CDKI can directly block the replication machinery by binding to proliferating cell nuclear antigen. Our data are in keeping with recent findings that TGFβ1 can induce p21 levels at both mRNA and protein levels (Akagi *et al.* 1996). Consistent with other reports, no change in p16 mRNA expression was found in response to TGFβ1 treatment.

Blocking new protein synthesis with cycloheximide prior to TGFβ1 exposure appears to show little effect on the TGFβ1-induced expression of p15, p21 and p27 mRNAs. This implies that the induction of these CDKIs by TGFβ1 does not require synthesis of new protein but induction probably occurs through the activation of a pre-existing signalling pathway, perhaps involving the Smad proteins. The results observed for p16 mRNA expression showing a rapid, and sustained, decrease in expression suggest that p16 mRNA has a very short half-life and regulation is independent of TGFβ1, but does require new protein synthesis.

Our findings demonstrate that TGFβ1 has a mainly inhibitory effect on prostate epithelium and that this inhibition is probably mediated in part by up-regulation of certain CDKIs, particularly p15. The inhibition of cell proliferation is achieved by placing cells in G1 and accompanied by up-regulation of Rb in its pRb form, a finding supported by measurable inhibition of cdk2 kinase. The role of TGFβ1 in prostate cell growth is uncertain, but altered expression and function of stromal cells may be an underlying mechanism of BPH. The increased levels of expression of TGFβ1 in prostate cancer are not associated with decreased cell proliferation and this finding needs further study to determine whether there is altered control of CDKIs, particularly in hormone unresponsive disease.

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