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Adenoviral-mediated overexpression of p16, p53 or TGFβ1 induces apoptosis of BPH cells

Research Article

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Abbreviations: adenovirus expressing p53, (Adp53); adenovirus expressing TGFβ1, (AdTGFβ1); benign prostate hyperplasia, (BPH); cytomegalovirus, (CMV); Dulbecco's modified Eagle medium, (D-MEM); glyceradehyde 3-phosphate dehydrogenase, (GAPDH); transforming growth factor β1, (TGFβ)

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Summary

Benign prostate hyperplasia (BPH), is the most commonly occurring benign disease in men older than 50 years of age. This report has provided a novel gene therapy strategy for BPH by adenoviral-mediated gene transfer of negative growth regulators p53, p16 and transforming growth factor β1 (TGFβ1). The effects of these adenoviral vectors on an established human BPH cell line, BPH-1, were examined. The apoptotic status of cells after p53, p16 and TGFβ1 expression was evaluated by DNA fragmentation, TUNEL (terminal deoxynucleotiyl transferase-mediated dUTP nick end labeling) staining, and flow cytometry. All these three gene products suppressed cell growth and induced apoptosis in BPH-1 cells. These results suggested that adenoviral-mediated gene transfer of p53, p16 and TGFβ1 may have a potential therapeutic application in treatment of BPH.

I. Introduction

Benign enlargement of the prostate, or benign prostate hyperplasia (BPH), is the most commonly occurring benign disease in aging men (Walsh, 1984). The incidence of BPH increases with advancing age, so that 50% of all men older than 50 years of age have symptoms arising from BPH (Walsh, 1984; McConnell, 1990). In over 25% of men over the age of 60, surgery is necessary (Walsh, 1984). Although these epidemiological facts underscore the importance of BPH as a major health problem, there is no specific or effective therapeutic modalities selectively targeted for BPH currently available (McConnell, 1990). Cell growth in the normal prostate is regulated by a delicate balance between cell death and cell proliferation. Disruption of the molecular mechanisms that regulate these two processes may underline the abnormal growth of the prostate gland leading to BPH (Kyprianou et al, 1996). Several genes which serve as growth negative regulators, or so-called gate keepers such as p16, p53, and TGFβ1, control cellular proliferation by either cell cycle arrest or induction of programmed cell death (apoptosis), and therefore, maintain the critical balance between cell proliferation and cell death in normal prostate homeostasis.

The p16 gene product, also called MTS1, INK4A, and CDKN2, is a cyclin dependent kinase inhibitor and an important negative cell cycle regulator whose functional loss may significantly contribute to malignant transformation (Serrano et al, 1993). Inactivation of p16 tumor suppressor gene is one of the most common events in a wide range of tumors including malignant melanoma (Talve et al, 1997), mesothelioma (Xiao et al, 1995), leukemia (Shapiro and Rollins, 1996), glioma, carcinomas of the lung, colon, liver, kidneys, esophagus, pancreas, breast, ovary, biliary tract and prostate, and sarcomas (Okamoto et al, 1994; Cairns et al, 1995; Liu et al, 1995; Smith-Sorenson and Hovig, 1995; Talve et al, 1997). These alterations in p16 may be a result of DNA mutations (Caldas et al, 1994; Kamb et al, 1994; Mori et al, 1994; Nobori et al, 1994), homozygous loss of p16 gene at the DNA level (Jen et al, 1994), or methylation of the promoter region at epigenetic level (Smith-Sorenson and Hovig, 1995). Restoration of p16 expression has resulted

in suppressed growth of a variety of tumor types including human prostate cancer cells (Gotoh et al, 1997), nonsmall cell carcinoma of the lung (Jin et al, 1995), glioma cells (Arap et al, 1995), prostate carcinoma (Gotoh et al, 1997) and hepatocellular carcinoma (Sandig et al, 1997), and others (Serrano et al, 1995; Quesnel et al, 1996; Schrump et al, 1996; Stone et al, 1996; Sandig et al, 1997). p53 is a nuclear phosphoprotein that acts as a transcription factor to control the expression of proteins involved in the cell cycle (Selter, 1994; Ozbun and Butel, 1995). In response to DNA damage, p53 accumulates in the nucleus and can arrest the cell cycle via the cyclin-dependent kinase inhibitor p21/Waf1. However, in neoplastic cells p53 usually induces apoptosis, or programmed cell death. Mutations in p53 have been reported in a majority of malignant cell types, and it has been estimated that p53 function is altered in half of all human malignancies (Nielsen and Maneval, 1998). Introduction of wild type p53 has been shown to inhibit the tumorigenic phenotype of many tumor cell lines (Nielsen and Maneval, 1998). TGFβ1, one of the most potent physiological negative regulators of epithelial cell growth, exerts its effect by binding to a cell surface receptor and triggering a signaling pathway that leads to the modulation of factors that directly regulate the cell cycle such as c-Myc, cyclins and cyclin-dependent kinases (Alexandrow and Mose, 1995). TGFβ1 in general stimulates mesenchyme and inhibits epithelial growth (Moses et al, 1990). Addition of TGFβ1 to cultured prostatic epithelial and stromal cells has been reported to inhibit proliferation in both cell types (Sutkowski et al, 1992). It is suggested that TGFβ1 may exert its growth inhibition via upregulation of other growth negative regulator genes such as p15 (Hannon and Beach, 1994) and p21 (Macleod et al, 1995). In this study, we examined whether the exogenous expression of the negative growth regulators p16, p53 and TGFβ1 will lead to growth inhibition of the BPH prostate.

II. Materials and methods

A. Generation of recombinant adenovirus Adp16, Adp53, and AdTGFβ1

Details of the construction of adenovirus expressing foreign cDNA gene have been previously described (Steiner et al, 2000). For construction of the adenovirus expressing wild type p16 (Adp16), the 960-bp human p16 cDNA gene was placed in an E1-deleted adenoviral shuttle vector under the control of the Rous sarcoma virus (RSV) promoter. The resultant adenoviral shuttle vector was cotransfected into 293 cells with pJM17, an adenoviral genome plasmid, by the calcium phosphate method (Kingston, 1993). The individual plaques were screened by direct plaque screening PCR method (Lu et al, 1998) using specific primers for the RSV promoter and for p16 the cDNA gene to identify the Adp16 plaque. The resultant Adp16 is a replication defective, recombinant adenoviral vector. Similarly, adenovirus expressing TGFβ1 (AdTGFβ1) was generated, in which TGFβ1 gene was modified so the resultant protein product TGFβ1 is an active form of TGFβ1 (Pierce et al, 1999). Adenovirus expressing wild type p53 gene under the control of the cytomegalovirus (CMV) promoter was a gift from University of Texas MD Anderson Cancer Center.

B. Adenovirus preparation, titration and transduction

Individual clones of Adp16, Adp53, and AdTGFβ1 were obtained by three-time plaque purification. Single viral clones were then propagated in 293 cells. The culture medium of the 293 cells showing a complete cytopathic effect was collected and adenovirus was purified and concentrated by twice CsCl2 gradient ultracentrifugation. The viral titration was performed as described (Graham and Prevec, 1991). Viral transduction was performed at various multiplicity of infection (moi) in a volume of 1 ml culture medium and incubated at 37^0C with gentle mixing every 15 min. After a 90-min incubation, the infectious supernatant was then replaced with fresh medium.

C. Cell culture and medium

Dulbecco's modified Eagle medium (D-MEM) and RPM1-1640 were purchased from Gibco BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). Human embryonic kidney 293 cells were grown in D-MEM with 10% heat inactivated FBS. The BPH-1 cell line, an immortalized but non-transformed human prostate-derived epithelial cell line (Hayward et al, 1995), was grown in RPMI-1640 medium with 10% FBS. All cell lines were grown in medium containing 100 units/ml penicillin, 100 μg/ml streptomycin at 37^0C in 5% CO_2.

D. Northern blot analysis

Cells were extracted and total RNA was isolated by RNeasy Total RNA Kit (Qiagen, Santa Clarita, CA). Total RNA (10 μg) was loaded on a 1.2% polyacrylamide gel and electrophoresed. The standard Northern blot transfer to a Nylon membrane (Hybond-N^+, Amersham Life Science, Buckinghamshire, England) was performed as previously described (Sambrook et al, 1989). The cDNA probe (p16, p53 and TGFβ1, respectively) was labeled by α-^{32}P-dCTP using random primer method (Prime-It II Kit, Stratagene, La Jolla, CA). The membrane was hybridized with the probe in Rapid-hyb buffer (Amersham Life Science) according to the manufacturer's protocol. The membrane was exposed to a Kodak X-ray film between two intensifying screens at -80^0C for autoradiography. The cDNA probe for glyceradehyde 3-phosphate dehydrogenase (GAPDH) was labeled as described above and used as an internal control for normalization.

E. ELISA assay

Cells were grown and maintained in the medium containing 10% charcoal-stripped FBS for this experiment. Supernatants were collected at 24 h, 48 h, 72 h, respectively, after viral transduction. The immunoassay for TGFβ1 production was determined by a Quantikine human TGFβ1 ELISA kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's manual.

F. Growth inhibition assay

Cells were plated at $5x10^3$ cells per well in 6-well plates; the next day the cells were incubated at 37^0C untreated or transduced by adenovirus. The cell numbers were counted in triplicate by a Coulter cell counter six days after transduction.

G. DNA extraction and gel electrophoretic analysis of DNA fragmentation

Soluble DNA was extracted as described previously (Oridate et al, 1996). Briefly, the cells floating in medium were collected at 48 h, 72 h and 96 h post transduction by centrifugation. The pellet was resuspended in Tris-EDTA buffer (pH 8.0). The cells were lysed in 10 mM Tris-HCl (pH 8.0), 10

mM EDTA, and 0.5% Triton X-100 on ice for 15 min. The lysate was centrifuged at 12,000xg for 15 min to separate soluble (fragmented) DNA from pellet (intact genomic) DNA. Soluble DNA was treated with RNase A (50 μg/ml) at 37^0C for 1 h, followed by treatment with proteinase K (100 μg/ml) in 0.5% SDS, at 50^0C for 2 h. The residual material was extracted with phenol/chloroform, precipitated in ethanol, and electrophoresed on a 2% agarose gel.

H. TUNEL assay

Slide flasks (NUNC, Roskilde, Denmark) were precoated with 50 μg/ml poly-L-lysine (Sigma, St. Louis, MO) for 15 min and washed twice with PBS. BPH-1 cells ($1x10^5$) were plated on slide flasks and grown for 24 h before viral transduction. The cells were then either untreated or transduced with control virus AdlacZ or Adp16, Adp53 and AdTGFβ1, respectively, at multiplicity of infection (moi) of 200. Cell monolayers grown on slides were rinsed twice with PBS at 72 h after transduction and subjected to TUNEL staining. *In Situ* Cell Death Detection Kit, POD (Boehringer Mannheim Corp., Indianapolis, IN) was used for the TUNEL assay according to the manufacturer's instruction and cells were visualized by fluorescence microscopy.

I. Flow cytometry to detect apoptotic population

Cells were arrested at G2/M phase by treating cells with 400 ng/ml nocodazole (Sigma) at 37^0C for 12 to 16 h (Park and Koff, 1998). After washing the cells, the synchronized cells were replated in fresh medium, and the cells were untreated or treated with viral transduction. Cellular DNA content that represents the cell cycle phase (G1, S, or G2/M) and apoptotic population (pre G1) was detected by flow cytometry via determination of propidium iodide staining (Lou and Xu, 1997; Zhang et al, 1999). After trypsinization, cells were washed with PBS and cell pellets were fixed in 70% ethanol at 4^0C overnight. After two washes with PBS, the cells were stained with 50 μg/ml propidium iodide (Sigma, St. Louis, MO), 10 μg/ml RNase A, 0.5% (v/v) Triton X-100 and 0.1% (w/v) sodium citrate for 30 min at room temperature in the dark followed by fluorescent flow cytometry.

III. Results

A. Adenoviral transduction resulted in overexpression of p16, p53 and TGFβ1 in BPH-1 cells

To determine whether the adenoviral vector served as a gene transfer vehicle that transduced BPH cells, an adenovirus carrying an E. coli lacZ reporter gene with a nuclear localization signal at the 5' upstream (AdlacZ) (previously (Oridate et al, 1996).) was used to transduce BPH-1. At a moi of 200, AdlacZ resulted in greater than a 95% transduction rate in BPH-1 cells as indicated by the percentage of cells that exhibited blue staining (not shown). To determine whether the adenovirus successfully transferred and expressed p16, p53 or TGFβ1, BPH-1 cells were transduced by adenovirus expressing p16 (Adp16) or TGFβ1 (AdTGFβ1) under the control of RSV promoter, or by adenovirus expressing p53 (Adp53) under the control of CMV promoter at moi of 200, respectively. The cell extracts were harvested 48 h after viral transduction and the mRNA was isolated. Under our experimental conditions, Northern blot analysis showed that there was no detectable endogenous p16, p53, and TGFβ1 in BPH-1 cells; however, there was a high exogenous mRNA expression of p16, p53 and TGFβ1, at 0.9 kb, 2.5 kb, and 2.5 kb transcript size, respectively, in the cells transduced by each corresponding adenovirus (**Figure 1**). In addition, culture media of BPH-1 control cells, cells transduced by AdlacZ control virus and AdTGFβ1 were collected at specific time points and analyzed for secreted TGFβ1 expression by an ELISA assay. As illustrated by **Table 1**, there was an increased TGFβ1 protein in the medium from AdTGFβ1 transduced BPH-1 cells that correlated with moi, except for the 72 h point with a moi of 500, which had an decreased TGFβ1 expression. This latter result was probably due to the direct cytoxicity of the adenovirus at this high moi which decreased cell number. In contrast, untreated BPH-1 cells and cells transduced by control virus did not show detectable TGFβ1 secretion. These

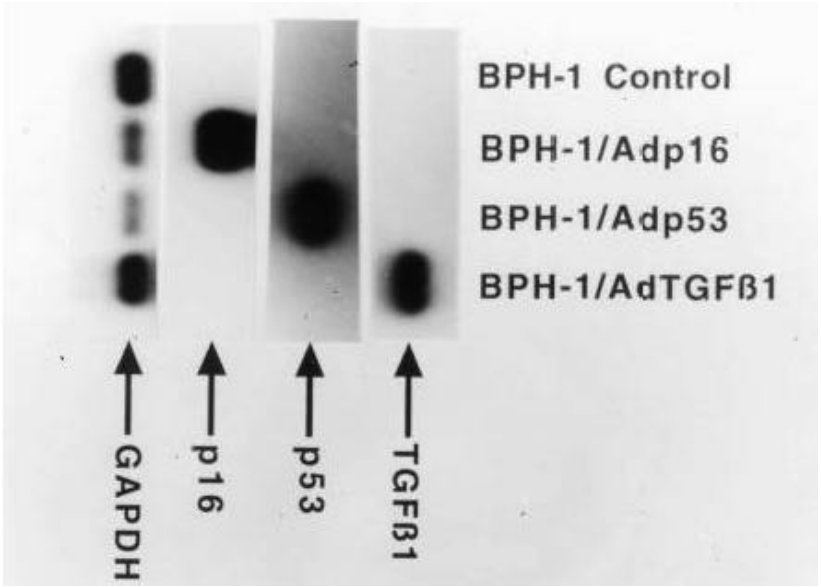

Figure 1. Expression of exogenous of p16, p53 and TGFβ1 by recombinant adenoviruses. BPH-1 cells transduced by Adp16, Adp53 and AdTGFβ1 at moi of 200 were harvested 48 h post transduction. Total RNA was extracted. Samples (10 μg/lane) were electrophoresed on a 12.5% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled p16, p53 and TGFβ1 cDNA probe (showed an 0.9 kb, 2.5 kb and 2.5 kb transcript, respectively). The Northern blot was then stripped and rehybridized with GAPDH cDNA (which showed a 1.2 kb transcript) to assess RNA integrity and gel loading..

Table 1. TGFβ1 production from BPH-1 cells

time	control cells	AdlacZ			AdTGFß1			
		100	200	500	100	200	500	(moi)
24 h	0	18.44±4.62	16.27±1.67	59.53±4.68	556.15±35.05	715.45±8.69		1045.74±247.46
48 h	33.98±5.03	23.86±5.96	20.25±0.85	N.D.*	1662.26±99.86	1964.66±160.52		2169.86±333.86
72h	42.17±1.02	72.67±1.70	50.97±18.10	72.06±5.96	3770.01±252.80	3178.77±26.06		1185.26±3.82

Cells were either untreated or transduced with AdTGFβ1 or control virus AdlacZ at various moi. Cell media were collected at various time points as indicated and the secreted amount of TGFβ1 was determined using an ELISA assay. The results were from three independent experiments each performed in triplicate and are represented as TGFβ1 (pg/ml) (mean±standard deviation).
*Not determined.

results demonstrate that adenoviral-mediated gene transfer successfully expressed the TGFβ1 transgene protein.

B. Adp16, Adp53, AdTGFβ1 inhibited BPH-1 cell growth

To determine the effects of p16, p53, and TGFβ1 on BPH cell growth *in vitro*, BPH-1 cells were either untreated or transduced with AdlacZ (control virus), Adp16, Adp53, and AdTGFβ1. Adp16, Adp53, and AdTGFβ1 significantly suppressed the growth of BPH-1 cells, with 84%, 46%, and 44% inhibition, respectively. Untreated and control virus treated cells had no significant inhibition (**Figure 2**).

C. Overexpression of p16, p53, and TGFβ1 induced apoptosis in BPH-1 cells

A potential mechanism by which these negative growth regulators inhibit BPH cell growth is by activation of programmed cell death. To investigate whether this was occurring, BPH-1 cells were transduced with Adp16, Adp53, and AdTGFβ1 or control virus AdlacZ. DNA was extracted from the cell medium (the supernatants which contained suspended cells) at 72 h post transduction for DNA laddering (fragmentation) analysis. As shown in **Figure 3**, the cells that were transduced by Adp16, Adp53, and AdTGFβ1 all had an apparent DNA laddering pattern-a hallmark of apoptosis-whereas untreated control cells and control virus treated cells had no significant DNA laddering. The data suggest that p16, p53 and TGFβ1 all were able to induce apoptosis in BPH-1 cells. TUNEL assay was also used to confirm that apoptosis occurred in the cells transduced by these adenoviruses. At 72 h after viral transduction, cells growing on culture dish were subjected to the TUNEL assay. There were more fluorescence-stained cells in Adp16, Adp53 and AdTGFβ1 transduced cells (**Figure 4B, 4C and 4D**) compared to untreated control (**Figure 4A**) or control virus transduced cells (not shown), indicating that

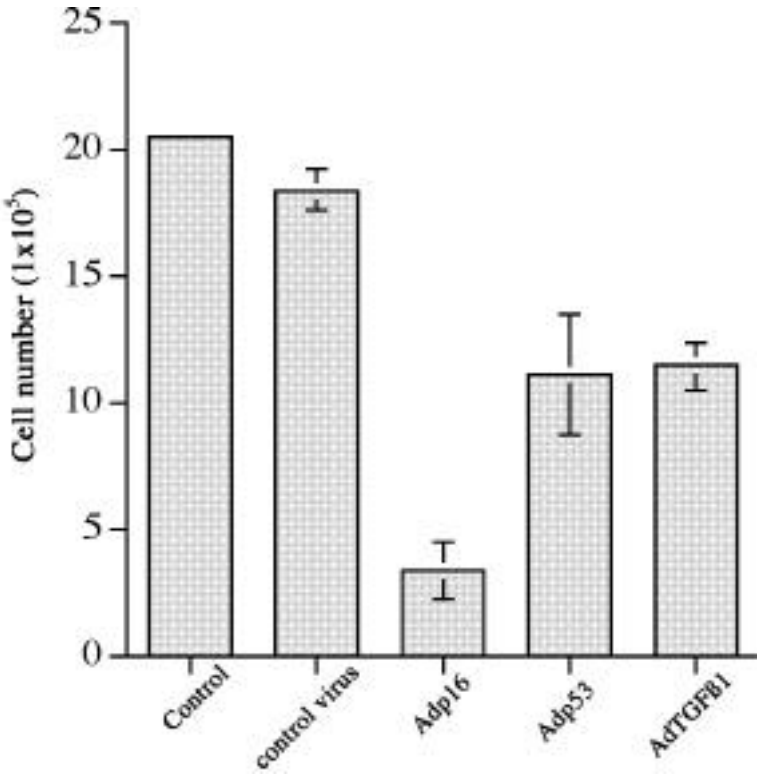

Figure 2. Inhibitory effects of p16, p53, and TGFβ1 on BPH-1 cell growth. BPH-1 cells were untransduced or transduced with Adp16, Adp53, and AdTGFβ1 or control virus AdlacZ at moi of 200. Cell numbers were counted at day 6 after viral transduction. The data represent the results from three independent experiments each performed in triplicate. *Error bars were too small to see in this figure.

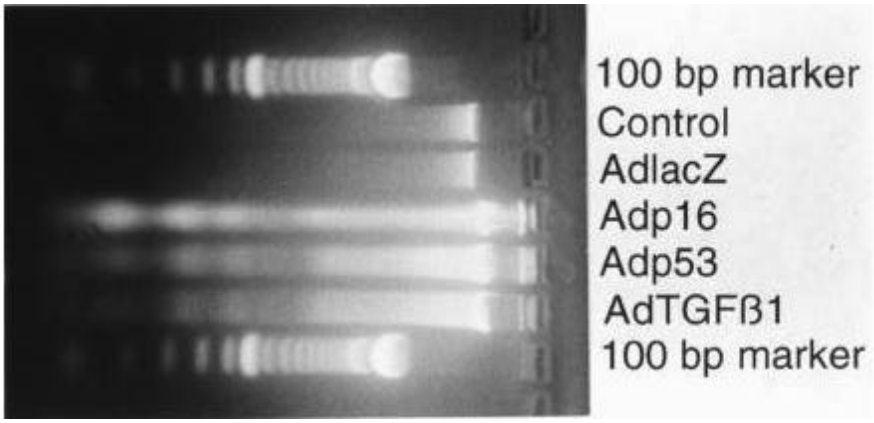

Figure 3. Overexpression of p16, p53 and TGFβ1 induces apoptosis in BPH-1 cells. Cells were untreated or transduced by either control virus AdlacZ or Adp16, Adp53 and AdTGFβ1 respectively at moi of 200, supernatants were collected 72 h post transduction. Soluble DNA was extracted from cell suspensions and electrophoresed on a 2% agarose gel.

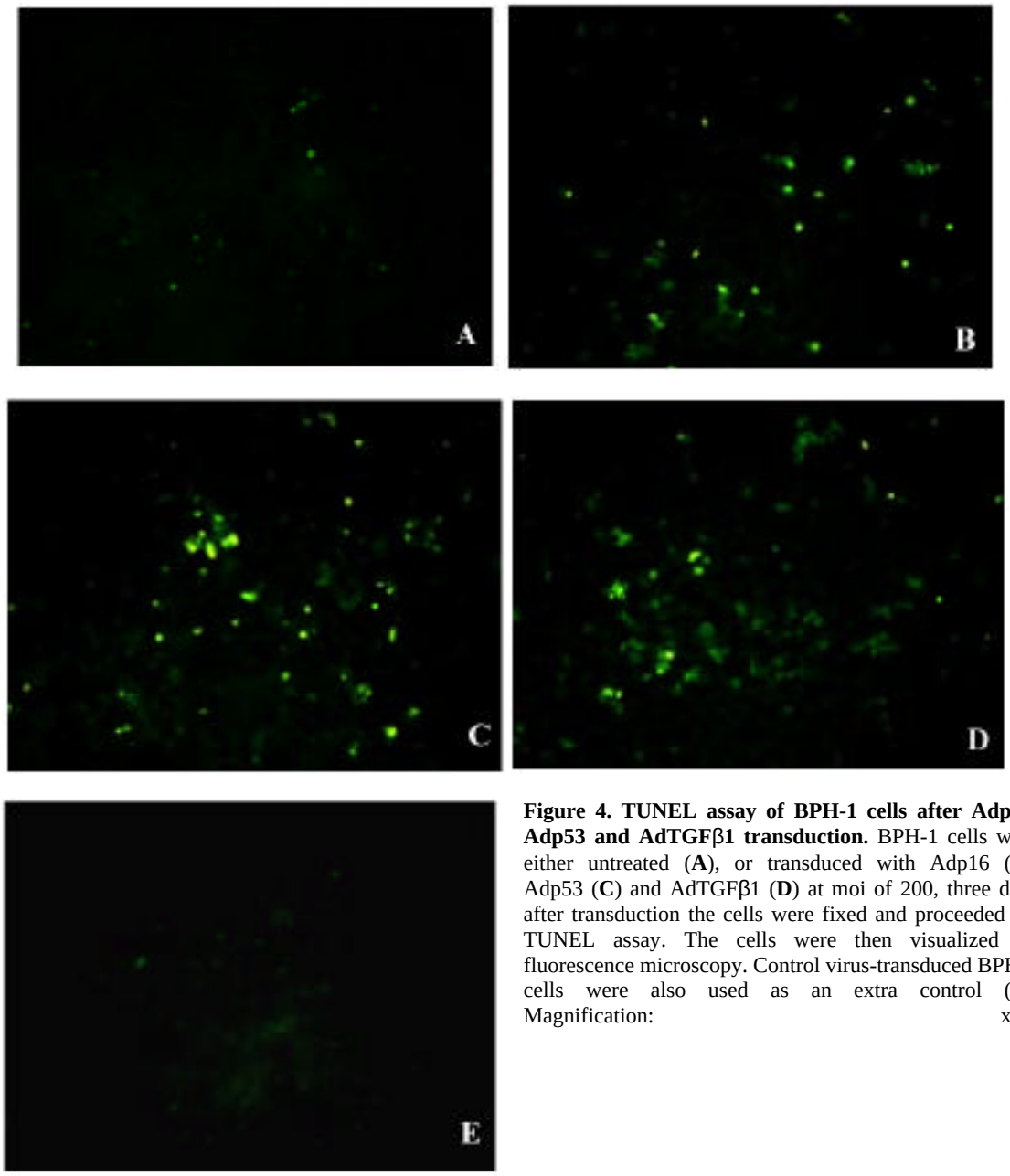

Figure 4. TUNEL assay of BPH-1 cells after Adp16, Adp53 and AdTGFβ1 transduction. BPH-1 cells were either untreated (**A**), or transduced with Adp16 (**B**), Adp53 (**C**) and AdTGFβ1 (**D**) at moi of 200, three days after transduction the cells were fixed and proceeded for TUNEL assay. The cells were then visualized by fluorescence microscopy. Control virus-transduced BPH-1 cells were also used as an extra control (**E**). Magnification: x20.

there were more apoptotic cells in Adp16, Adp53 and AdTGFβ1 treated cells than the control or control virus transduced cells. In addition, low background apoptosis was observed in untreated control cells (**Figure 4A**). To quantitate the apoptotic cell population, cells were synchronized first at G2/M phase arrest by treatment with nocodazole and then transduced with these adenoviruses. Cell populations at different phases were analyzed by flow cytometry using propidium iodide staining. As shown in **Figure 5** and **Table 2**, at 72 h post viral transduction, untreated and control virus treated cells had a very small populations of apoptotic cells, with a pre-G1 phase population of 1.32% and 2.0%, respectively. In contrast, cells transduced with Adp16, Adp53, or AdTGFβ1, all had a significantly larger apoptotic cell population, with the highest apoptotic population observed by Adp53 (46.76%), followed by AdTGFβ1 (35.06%), and lowest population observed by Adp16 (31.35%). These results again demonstrated that overexpression of p16, p53 and TGFβ1 lead to apoptosis in BPH cells. While all these cells had a similar percent of population in S phase, cells transduced by adenoviruses expressing p16, p53 and TGFβ1 had a much less cell population in both G1 and G2 phases compared to untreated and control virus treated cells (**Figure 5 and Table 2**), indicating that cells transduced by all these three negative growth control genes had a much reduced population for cell division and growth compared to that of untreated and control virus treated cells.

IV. Discussion

In this study, we proposed to use a gene therapy approach by applying adenoviral vectors expressing three so-called negative growth regulators, p16, p53, and TGFβ1 for BPH treatment. Gene therapy strategies to use critical tumor suppressor genes/negative growth regulators are effective against many cancers. Adenoviral-mediated p53 gene therapy has shown inhibition in colorectal cancer (Spitz et al, 1996), prostate cancer (Yang et al, 1995; Ko et

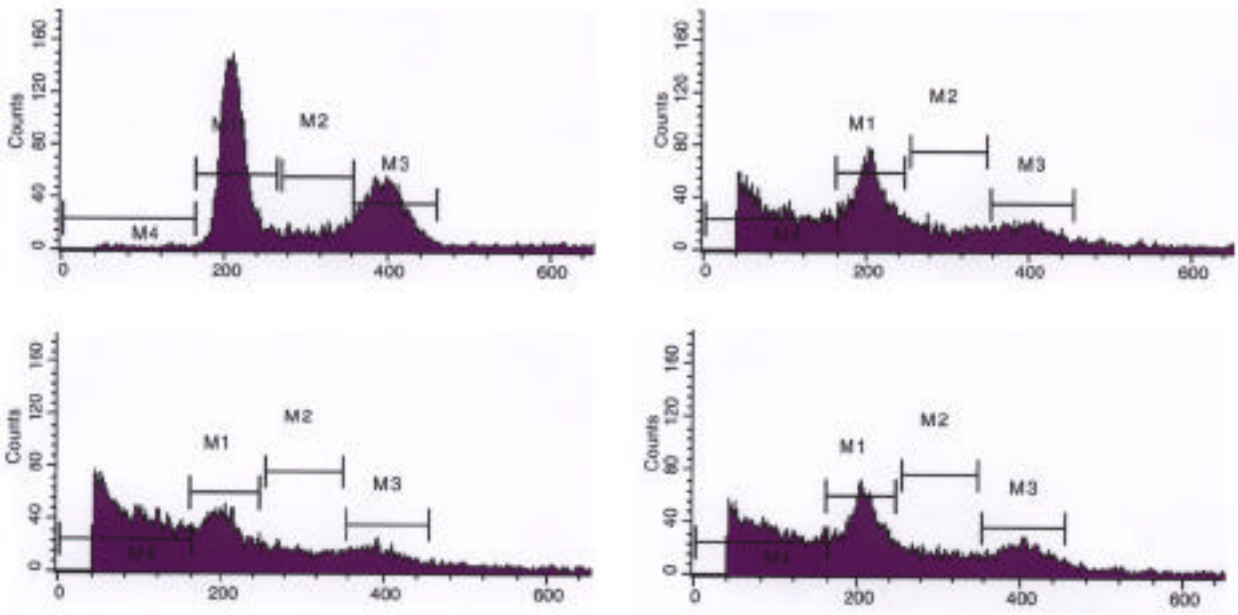

Figure 5. DNA content distribution of BPH-1 cells. Cells were either untreated or transduced with various adenoviruses at moi of 200. Both attached cells and cells in suspension were collected 72 h post viral transduction and processed for fluorescent flow cytometry. Shown are untreated control cells (**A**), cells transduced by Adp16 (**B**), Adp53 (**C**) and AdTGFβ1 (**D**). Cells transduced by control virus showed the similar results as in (**A**) (not shown and also see Table 2). M1: G0/G1 phase, M2: S phase, M3; G2/M phase, M4: pre-G1

Table 2. Cell population analysis by flow cytometry

cells	pre-G1/apoptosis (%)	G0/G1 (%)	S (%)	G2/M (%)
untreated	1.32	49.42	10.33	28.34
control virus	2.00	54.25	10.59	25.92
Adp16	31.35	32.64	13.11	13.58
Adp53	46.76	25.18	11.11	10.28
AdTGFβ1	35.06	29.05	12.58	14.86

$1x10^6$ BPH-1 cells were either untreated or transduced with various adenoviruses at moi of 200. Both attached cells and cells in supernatant were collected 72 h post viral transduction, stained with propidium iodide and processed for fluorescent flow cytometry. The percentage of cell population (from two independent experiments) at each cell phase was presented.

al, 1996), breast cancer (Nielsen et al, 1997; Xu et al, 1997), cervical cancer (Hamada et al, 1996), ovarian carcinoma (Mujoo et al, 1996), and melanoma (Cirielli et al, 1995). A retroviral vector expressing BRCA1 tumor suppressor gene was used in Phase I human clinical trial for advanced prostate cancer (Steiner et al, 1998). Adenovirus expressing p16 gene significantly inhibited prostate cancer growth and prolonged survival in an animal model (Steiner et al, 2000). Therefore, it is possible that adenoviral vectors expressing p16, p53, and TGFβ1 may have a therapeutic potential for BPH therapy.

To determine whether overexpression of these negative growth regulators would lead to cell growth inhibition, recombinant adenoviruses expressing p16, p53, and TGFβ1 were used to transduce an established human BPH cell line, BPH-1, and the potential inhibitory effect was evaluated. The expression of exogenous p16, p53, and TGFβ1 via adenoviral-mediated gene transfer inhibited cell growth and induced apoptosis in BPH-1 cells. Interestingly, while p16 showed the strongest inhibitory effect on BPH-1 cell growth among the three negative growth regulators (**Figure 2**), it did not appear to lead to the highest apoptotic population (**Figure 5 and Table 2**). One explanation is that p16 may have dual inhibitory mechanisms in BPH-1 cells, composed of apoptosis and other inhibitory mechanisms such as cell cycle arrest. As p16 has been reported previously to be able to induce senescence (Kingston, 1993; Steiner et al, 2000), the observed growth inhibition by Adp16 in BPH-1 cells may be a result of the combination of apoptosis and senescence. Moreover, our recent studies showed that p16 inhibits VEGF expression and suppresses neovascularization and angiogenesis in breast cancer cells (Lu *et al*., Cancer Therapy, 1: 143-151, 2003) and prostate cancer cells, as well as in BPH-1 cells (our unpublished data). As VEGF and other angiogenic factors play a major role on cell proliferation and survival, the anti-VEGF effect renders another level of p16's anti-cell growth function. Thus, p16 can cause cell-cycle arrest, induce apoptosis, senescence and anti-angiogenesis, which may account for it being as the most potent cell-growth inhibitor (**Figure 2**), as cell growth result is a net result of the combination of all these effects. While some reports showed that p53 is a more potent cell killer, the seemingly less potent p53 cell-killing effect in our study may be cell specific in this case. The BPH-1 cells was established by immortalizing human primary prostate epithelial cells with SV40 large T antigen, BPH-1 line has an increased endogenous levels of p53 (Hayward et al., 1995), which may desensitize Adp53 effect in BPH-1 line as it already has a basal level of p53. On the other hand, Adp53 clearly demonstrated a greater effect on apoptosis in our study, as reflecting by flow cytometry data (**Figure 5 and Table 2**, see pre-G1/apoptosis cell population), which is consistent with other reports.

The current therapies for BPH such as prostatectomy are invasive with side effects. Furthermore, the current therapies cannot arrest BPH progression. We hypothesize that molecular therapies may be less toxic and invasive and potentially more effective. Overexpression of p16, p53 and TGFβ1 leads to suppression of BPH cell growth and even induction of apoptosis, suggesting that this approach may be useful as gene therapy to reduce BPH and relieve urinary obstruction. Whether the combined use of two or more of these adenoviruses on BPH cells will lead to a potential synergistic inhibitory effect needs to be further examined. To our knowledge, this is the first report to use a gene therapy approach evaluate its potential to treat BPH disease. These studies support further studies using *in vivo* models such as BPH dogs followed by prostate volume measurement as a treatment endpoint.

Acknowledgments

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