Gene Therapy of Prostate Xenograft Tumors with a $p75^{NTR}$ Lipoplex

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Abstract. *The p75neurotrophin receptor ($p75^{NTR}$) has been characterized as a tumor suppressor in human prostate cancer. Ectopic re-expression of this cell surface receptor protein in prostate cancer cell lines has been shown to increase the frequency of apoptosis and concurrently reduce the rate of cellular proliferation. Since it is difficult to maintain a therapeutic level of this membrane receptor protein by systemic administration in the blood, we utilized an alternative gene therapy strategy based upon intra-tumoral injection into xenografts of PC-3 prostate tumor cells of a lipoplex containing the $p75^{NTR}$ gene. Administration of the $p75^{NTR}$ gene into subcutaneous PC-3 xenografts suppressed in a dose-dependent manner the growth of tumors. Within the gene therapy-treated tumors, re-expression of the $p75^{NTR}$ gene product was associated with increased apoptosis and reduced proliferation of tumor cells. These results suggest that the $p75^{NTR}$ may be a suitable candidate for gene therapy of prostate cancer cells.*

Gene therapy is based upon the requirement that a gene with potential therapeutic effects be able to produce a key protein where it is needed (1). In a number of illnesses, including cancer, the expression of regulatory genes may be modified, altering levels of the gene product to the extent that it no longer maintains homeostatic function within the cell. Tumor suppressor gene products are typically suppressed in many cancers. Ectopic re-introduction of a tumor suppressor gene with subsequent production of the protein has been studied as a potential means for gene therapy of some tumor cells. The use of non-viral cationic lipoplexes has proven to be an efficient method to deliver genes of interest to cells both *in vitro* and *in vivo* (2, 3). Liposomal vectors provide distinct advantages over recombinant viral vectors because they are nonpathogenic, less immunogenic and simple to prepare. Consequently, lipid-mediated gene transfer into tumor cells facilitates the characterization of candidate genes for the development of cancer gene therapies.

The p75 neurotrophin receptor ($p75^{NTR}$) protein has been well characterized in the nervous system and, more recently in a wide variety of peripheral organs and tissues (4, 5). It has been identified as a member of the TNF receptor superfamily based upon a similarity of intracellular death domain sequences (4, 7). However, unlike other TNF receptor superfamily members, $p75^{NTR}$- mediated signal transduction can occur in a ligand-independent manner (8, 9), as appears to be the case in the prostate and bladder (10, 11). This allows for the expression of $p75^{NTR}$ to be the determinant in signal propagation as opposed to the requirement of ligand binding. In the prostate, $p75^{NTR}$ is normally expressed in epithelial cells where, upon transformation, expression is progressively lost with progression of the cancer cells to a more malignant pathology (5, 12). The $p75^{NTR}$ has been characterized as a tumor suppressor (13) and metastasis suppressor (14). Its re-expression in tumor cells reduces growth by suppressing proliferation and increasing the rate of apoptosis (13, 14). In this context, re-expression of $p75^{NTR}$ has been shown to modulate several cell cycle effector proteins associated with proliferation (10) as well as to induce caspase-mediated apoptosis *via* mitochondrial effectors (11). Xenografts in SCID mice of cells stably transfected to express in rank-order, low, intermediate and high levels of $p75^{NTR}$ yield smaller tumors, respectively, than those established by control cell xenografts (13). However, the effect of re-introducing $p75^{NTR}$ to an established tumor has yet to be determined. Hence, in this report, we demonstrate that $p75^{NTR}$ is a potent and efficient agent for gene therapy directed toward prostate cancer cell tumors. By intratumoral injection of lipoplexes containing cDNA for $p75^{NTR}$, human prostate cell tumors established in mice showed marked reduction in growth rate and subsequent tumor size. Together these results not only reveal, but also define, parameters in which $p75^{NTR}$ is a novel and effective candidate for gene therapy treatment of prostate cancer.

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Materials and Methods

Cell culture conditions. The prostate tumor cell line PC-3 was obtained from the American Type Tissue Culture Collection (Rockville, MD, USA) (13). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech Inc., Herdon, VA, USA) containing 4.5 g/L glucose and L-glutamine supplemented with 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA), and antibiotic/antimycotic (100 units/mL penicillin G, 110 μg/mL streptomycin, 0.25 μg/mL amphotercin B; Mediatech). Cells were grown at 37˚C in 10% CO_2.

Tumor growth and injections. Cells were harvested and washed with PBS. One million cells resuspended in serum containing media (50 μL per mouse; Mediatech) and Matrigel (Collaborative Biomedical, Bedford, MA, USA) (1:1) were injected subcutaneously into the flanks of SCID mice (4 per experimental group; Taconic, Germantown, NY, USA). Intratumoral vehicle and/or vector injections began 5 days after tumor cell injection (10 days in $p75^{NTR}$ *vs.* Neo experiment; 24 days in delay injection experiment). The vehicle used for all experiments was lipofectamine obtained from Invitrogen Life Technologies (Carlsbad, CA, USA) at a concentration of 1 nM lipofectamine/1 μg DNA in TE buffer per injection per mouse for best efficiency with least toxicity (15). Lipid vehicle and DNA were combined in serum-free media to a total volume of 50 μL and incubated for 30 minutes before intratumoral injection. The DNA vectors were pCMV5A ($p75^{NTR}$ cDNA) and pCMVneo generously provided by Barbara Hempstead (13) and used according to the parameters described in the text. Tumor volumes were measured prior to each injection of lipoplex and calculated using the formula π/6 x L x W x H (14, 16).

Immunohistochemistry. Following the completion of injections, control, vehicle alone and $p75^{NTR}$- treated tumors were excised from the flanks of mice, fixed in 10% formalin, embedded in paraffin wax and sectioned to 5 μm thickness. In preparation for staining, tissue sections were deparaffinized by washing 2 times for 5 minutes in xylene, followed by a 5-minute washes in a graded series of ethanol solutions (100%, 90%, 80%, 70%), and finally washed 3 times for 5 minutes each in TBS. Three adjacent sections were selected for staining with anti-$p75^{NTR}$ (Upstate cell signaling solution, Lake Placid, NY, USA), anti-phospho-histone H3 (P-histone H3) (Upstate), or detection of apoptosis (Fluorescein-fragEL DNA fragmentation detection kit; Oncogene Research Products, San Diego, CA, USA). In the case of apoptosis detection, staining was performed according to the manufacturer's protocol. For P-histone H3 staining, sections were further permeablized using proteinase K (Oncogene) according to the manufacturer's instructions. For staining of both $p75^{NTR}$ and P-histone H3, primary antibodies in TBS (1:100) were incubated on tissue sections for 1 hour in a humidified incubator at 27˚C, followed by washing 3 times in TBS. Secondary FITC-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were used at 1:100 in TBS, incubated at room temperature in the dark for 1 hour. After washing 3 times in TBS, coverslips were mounted and sections were visualized at 20X using fluorescent microscopy (Zeiss axioplan 2).

Statistical analysis and graphs. The statistical differences between two specific groups were analyzed by the *t*-test and differences between data sets were analyzed for ANOVA using the Prizm program (Graph Pad Software). Data expressed as the mean±SEM was considered significant when $p<0.05$. All graphs were plotted using the Prizm program.

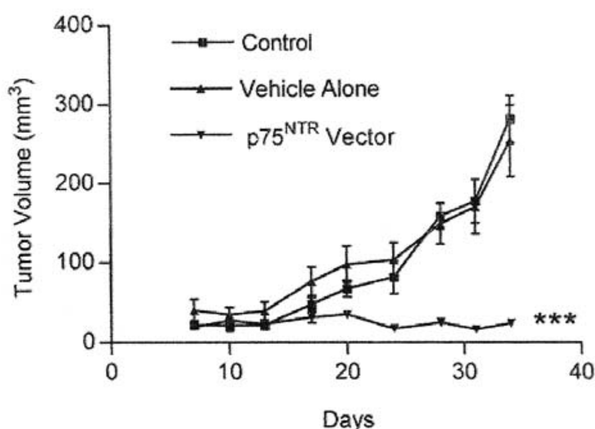

Figure 1. *PC-3 tumor volumes following injection with $p75^{NTR}$ lipoplex (vehicle + $p75^{NTR}$ vector), injection with empty liposome (vehicle alone), or no injection (control). *** $p<0.001$.*

Results

Intratumoral injections of $p75^{NTR}$ DNA suppress growth. Since transformed prostate cells no longer express detectable levels of $p75^{NTR}$ (12), and stable reintroduction of the protein to cancerous cells results in their reduced proliferation and increased apoptosis (13), we examined the possibility that $p75^{NTR}$ may be a suitable candidate for gene therapy. Hence, PC-3 prostate tumor cells were subcutaneously injected into the flanks of SCID mice where they were allowed to grow prior to any additional treatments (control). After 5 days post-seeding of the tumor cells, treatment of a subset of the subcutaneous tumors was initiated and continued three times per week by intratumoral injections with the empty vehicle (vehicle alone), or by intratumoral injections with the vehicle coupled with 10 μg $p75^{NTR}$ DNA (vehicle + vector) over a period of approximately five weeks. The subcutaneous tumors treated with the $p75^{NTR}$ DNA were significantly ($p<0.001$) smaller than tumors of the control group and the vehicle alone group (Figure 1). The tumors that grew in the control and vehicle alone groups were not significantly different from each other (Figure 1). Moreover, relative to the vehicle alone group, intratumoral injections with increased amounts of $p75^{NTR}$ DNA reduced tumor growth in a dose-dependent manner (Figure 2). Beginning on day 5 following subcutaneous tumor cell inoculation, relative to vehicle alone, intratumoral injections with 1 μg $p75^{NTR}$ DNA significantly ($p<0.05$) reduced tumor growth. Similarly, relative to vehicle alone, intratumoral

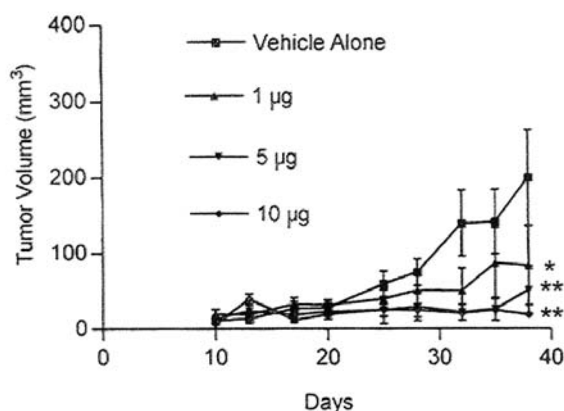

Figure 2. *Dose-dependent effect upon PC-3 tumor volumes following injection with 1, 5, or 10 mg p75^{NTR} cDNA contained in liposome, or empty liposome (vehicle alone). * $p<0.05$. ** $p<0.01$.*

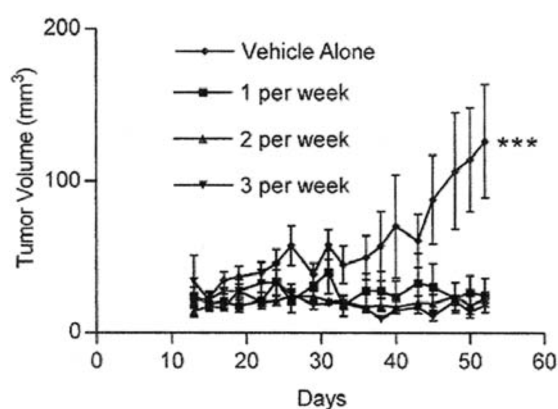

Figure 3. *Time-dependent effect upon PC-3 tumor volumes following injection 1, 2, or 3 times per week with p75^{NTR} cDNA contained in liposome or empty liposome (vehicle alone). *** $p<0.001$.*

injections with 5 μg or 10 μg p75^{NTR} DNA also significantly ($p<0.01$) reduced tumor growth. Since treatments with 5 μg or 10 μg p75^{NTR} DNA were not significantly different from each other in reducing tumor growth (Figure 2), it was determined that 5 μg p75^{NTR} DNA was sufficient to maximally suppress tumor growth over a duration of approximately five weeks.

Frequency of treatment with p75^{NTR} DNA to inhibit tumor growth. In order to further define the parameters of effective p75^{NTR}-mediated tumor growth inhibition, the frequency of injections of p75^{NTR} DNA was varied from between one to three per week. Treatment with 5 μg p75^{NTR} DNA once, twice or three times per week all significantly ($p<0.001$) reduced tumor growth compared to vehicle alone (Figure 3). Since all three treatment intervals resulted in relatively identical tumor growth suppression, it was concluded that 5 μg p75^{NTR} DNA injected once per week was sufficient to achieve maximum growth inhibition.

Delayed treatment with p75^{NTR} reduces tumor growth in established tumors. Using an optimized treatment regimen of 5 μg p75^{NTR} DNA injected once per week, we next sought to determine if p75^{NTR} DNA treatments could retard the growth of a well-established tumor. The subcutaneous PC-3 tumors were allowed to grow for twenty-four days, at which point large palpable tumors were treated by intratumoral injection with 5 μg p75^{NTR} DNA injected once per week. Treatment with p75^{NTR} DNA resulted in tumors that were at least two and a half times smaller ($p<0.05$) compared to the vehicle alone control group (Figure 4). This suggests that p75^{NTR} treatments are not only effective during early tumor formation, but can also suppress the growth of established tumors.

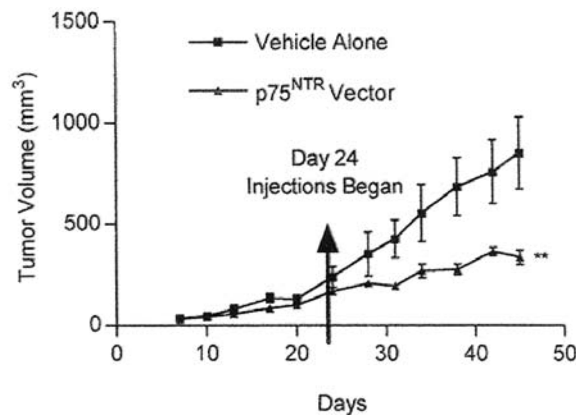

Figure 4. *PC-3 tumor volumes following injection with p75^{NTR} cDNA contained in liposome (p75^{NTR} vector) or empty liposome (vehicle alone). Injections began 24 days after initial tumor cell injections allowing for further establishment of primary tumor. ** $p<0.01$.*

Comparison of treatments with p75^{NTR} DNA and empty vector. Treatment by intratumoral injection of p75^{NTR} DNA appears to be effective in reducing the growth of both nascent and established tumors. In order to confirm that the observed results were a function of the tumor suppressor activity of the p75^{NTR} gene product, an empty vector that did not contain the p75^{NTR} DNA coding sequence was compared to the vector containing the p75^{NTR} DNA sequence for their ability to suppress tumor growth under optimized experimental conditions. Clearly, tumors treated with the vector-containing p75^{NTR} DNA were able to significantly ($p<0.01$) suppress growth to a greater extent than empty vector treated tumors (Figure 5). These results

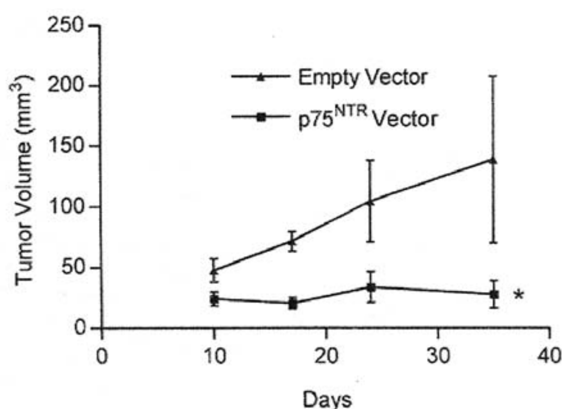

Figure 5. *PC-3 tumor volumes following injection with p75^{NTR} cDNA contained in liposome (p75^{NTR} vector) or with all vector components except for p75^{NTR} coding sequence (empty vector). * $p<0.01$.*

confirm that the tumor suppressor effect of the vectors is primarily associated with the coding sequence of the p75^{NTR} DNA.

Treatment of subcutaneous tumors with p75^{NTR} DNA results in p75^{NTR} protein expression, increased numbers of apoptotic cells and a reduction in the number of proliferative cells. In order to determine the effect of treating tumor cells with p75^{NTR} DNA, we examined adjacent sections of PC-3 tumor for expression of the p75^{NTR} gene product as well as markers for apoptosis and proliferation. As expected, since the original cell line has lost p75^{NTR} protein expression (12), the control PC-3 tumor expressed no detectable p75^{NTR} gene product (Figure 6A). Treatment with p75^{NTR} DNA resulted in localized expression of p75^{NTR} (Figure 6B), most probably in the vicinity of a recent injection sight. Similarly, when tumor sections were examined by TUNEL analysis, control sections showed infrequent apoptotic cells (Figure 6C). In tumors treated with p75^{NTR} DNA, a localized area coinciding with the area of p75^{NTR} protein expression contained a large number of apoptotic cells far in excess of the control sections (Figure 6D). In order to examine the incidence of proliferating cells in the tumors, immunofluorescence of P-histone H3 was used as a marker for cells undergoing mitotic division (17, 18). In the case of control tumors, the P-histone H3 protein was uniformly expressed throughout the tumor (Figure 6E). In contrast, in the tumors treated with p75^{NTR} DNA, the region coinciding with p75^{NTR} protein expression (Figure 6B) showed a paucity of P-histone H3 protein-stained cells (Figure 6F). The cells that were positive for proliferation appeared to be confined to an area that was outside the region expressing p75^{NTR} (white), as well as the region that expressed apoptotic cells (green). These results show that treatment of tumors with p75^{NTR} results in the expression of the p75^{NTR} gene product, a coinciding region of apoptosis, as well as a reduction in proliferative cells.

Discussion

Recently a group of cell surface receptor proteins with common sequence motifs were identified as members of the TNF receptor superfamily. Members of this group include p75^{NTR}, TNFR1 and the Fas receptor, among others (19, 20). As a consequence of the function of these proteins to regulate apoptosis, substantial interest has emerged regarding their therapeutic potential. For instance, it has been shown that gene therapy-based introduction of Fas induces apoptosis in gliomas and osteocarcinoma (21, 22). Similarly, introduction of a constitutively active TNFR1 exhibited antitumor activity in melanoma (23). However, no known studies have been performed to date that examine the potential of the p75^{NTR} as a gene therapy agent. Significantly, this receptor protein is of particular interest because, in specific cell types, expression of the p75^{NTR} is independent of ligand presence or interaction, where it is sufficient for the induction of apoptosis (8, 24, 25).

The p75^{NTR} is a tumor suppressor in prostate and bladder cancer cells where inhibition of growth is achieved through induction of apoptosis and inhibition of proliferation (13, 17, 1). Since the suppression of growth in these cancer cells is correlated with the dose-dependent expression of the p75^{NTR} protein (13), it seemed plausible that p75^{NTR} could be a candidate for gene therapy applications by ectopic re-expression in cancer cell xenografts. Nonviral gene delivery approaches using complexes of cationic lipids and plasmid DNA (lipoplexes) have distinct advantages for gene therapy applications in that lipoplex treatments are generally nonpathogenic, significantly less immunogenic and are relatively simple to prepare for application (26). Furthermore, cationic liposome complexes have been shown to be effective gene delivery vehicles in both xenograft tumor models (27) as well as in clinical trials (28). Consequently, in order to test the efficacy of p75^{NTR} as an agent for gene therapy, a DNA/lipoplex was injected into subcutaneous tumors of PC-3 cells grown in SCID mice. Significantly, xenografts of PC-3 tumor cells treated with a p75^{NTR} lipoplex were growth suppressed relative to control treatments. Moreover, this effect was dose-dependent with increased amounts of p75^{NTR} lipoplex providing the greater inhibition of xenograft growth. Since treatments with either 5 μg or 10 μg DNA were similar in sustaining maximal inhibition of tumor growth, it was determined that 5 μg was the minimal optimal dose to sustain tumor growth inhibition. Additionally, the interval between treatments with 5 μg

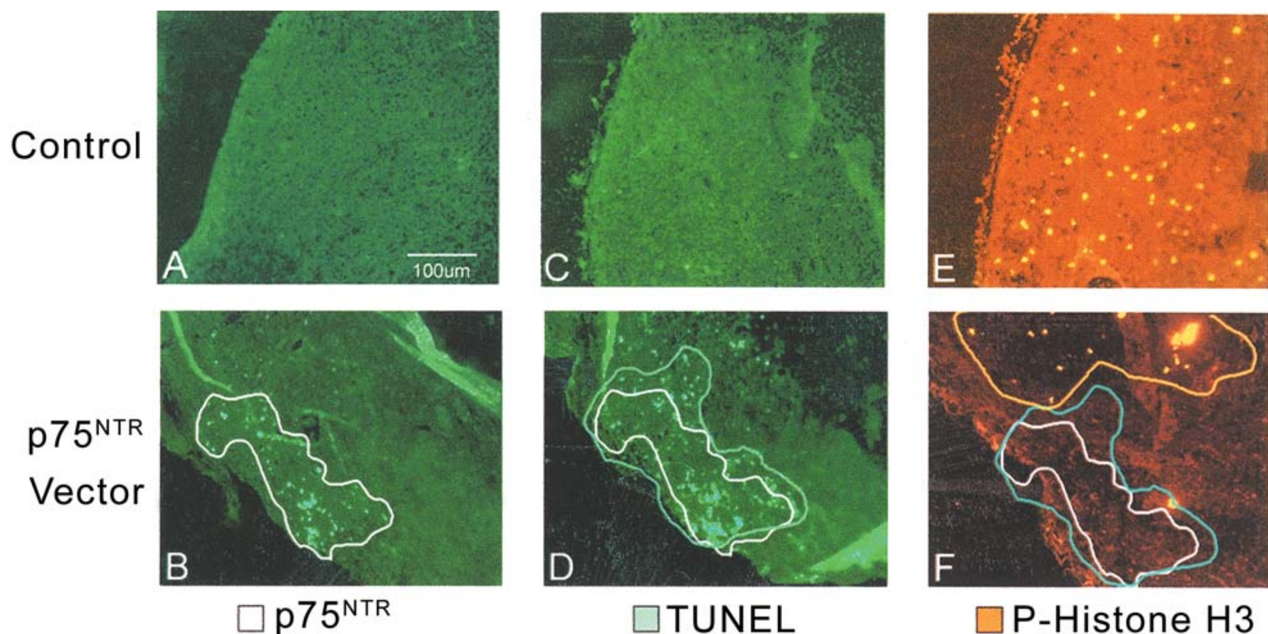

Figure 6. *Adjacent tumor sections derived from control PC-3 tumors or tumors treated with p75^{NTR} lipoplex. Sections were analyzed for expression of p75^{NTR} (region marked in white), TUNEL (region marked in green), or expression of P-histone H3 (region marked in orange).*

p75^{NTR} was examined to take account of continually proliferating tumor cells that may escape p75^{NTR}- mediated gene therapy of xenografts. Significantly, treatment with the p75^{NTR} lipoplex once per week was equally effective in suppressing xenograft growth as more frequent treatments, suggesting that the p75^{NTR} lipoplex may provide a relatively durable treatment for gene therapy suppression of prostate cancer cell growth.

Since p75^{NTR} lipoplex gene therapy was effective in suppressing early growth of PC-3 prostate tumors, the treatment of larger tumors established from 24 days of uninterrupted growth (volume of approximately 200 mm^3) was also examined for efficacy of the p75^{NTR} lipoplex. Following the establishment of larger tumors (24 days growth), with subsequent treatment for 25 days, lipoplex-treated tumors were approximately two and a half times smaller than control-treated tumors (Figure 4). Significantly, p75^{NTR} lipoplex gene therapy did not suppress large tumor growth to the same extent as early growth tumors. Whereas parameters optimized for gene therapy treatment of early growth tumors may be effective in suppressing the progressive growth of smaller tumors, the p75^{NTR} lipoplex may not affect all the cells of a larger tumor, so that those parts of the large tumor not affected by the gene therapy treatment may continue the growth of the tumor unabated.

Macroscopically, gene therapy with a p75^{NTR} lipoplex had a substantial effect upon reducing overall tumor growth. Immunostaining for p75^{NTR} protein revealed very little expression in control PC-3 tumors (Figure 6A), whereas localized expression was observed in treated tumors (Figure 6B). Since these growth-suppressed tumors were treated with the p75^{NTR} lipoplex for a period of over 30 days, many, if not most, of the tumor cells that had expressed the p75^{NTR} protein from previous treatments would have undergone apoptosis, as shown in some localized regions (Figure 6D), and subsequently have been eliminated from the tumor mass. The region of xenograft tumor expressing high amounts of p75^{NTR} protein (Figure 6B) most probably is an area that was subjected to p75^{NTR} lipoplex treatment relatively recently before the excision of the tumor. Significantly, the area of xenograft tumor exhibiting apoptotic nuclei (Figure 6D) clearly overlapped with the area of p75^{NTR} protein expression (Figure 6B), suggesting that p75^{NTR} expression was inductive of the apoptosis. Analysis for DNA fragmentation showed a very low incidence of apoptotic cells in control tumors (Figure 6C) and, again, regional high levels of apoptosis in p75^{NTR}-treated tumors (Figure 6D). Interestingly, the area exhibiting apoptotic nuclei (Figure 6D) was greater than the area exhibiting p75^{NTR} expression (Figure 6B). This suggests a bystander effect (29), where collateral induction of apoptosis was induced in cells adjacent but outside the immediate vicinity of p75^{NTR} expression (Figure 6D). Since growth is the net result of proliferation minus apoptosis (30, 31), we also examined regions of cellular proliferation within the xenograft

tumors by immunostaining for P-histone H3. The P-histone H3 protein is a mitosis marker present in cells in the M-phase of the cell cycle (32) and is regarded as one of the more accurate indicators for proliferating cells (33). In control tumors, expression of P-histone H3 was uniform and widely expressed throughout the tissue section (Figure 6E). Conversely, in $p75^{NTR}$- treated tumors, expression of P-histone H3 was much more sporadic (Figure 6F). In addition, the region in which P-histone H3 was detected in $p75^{NTR}$ lipoplex-treated tumors was outside the region in which $p75^{NTR}$ had been re-expressed as well as outside those regions positive for apoptosis (Figure 6F). Together these results indicate that, in control xenograft tumors, $p75^{NTR}$ is not expressed, proliferation is widespread and background apoptosis occurs at a relatively low level, leading to unregulated growth of the tumor mass. Conversely, gene therapy of xenograft tumors with a $p75^{NTR}$ lipoplex produced regional expression of the $p75^{NTR}$ that was coincident with a high incidence of apoptotic cells and absence of proliferating cells, that resulted in reduced growth of the tumor mass.

Recently, the potential for accumulation of mutations in plasmid DNA used for non-viral gene therapy was elucidated in the context of the gene amplification process in the bacteria and target cell (6). Such modifications in nucleotide sequence could have particularly adverse effects where the gene therapy was intended to restore function and concurrently maintain viability of the target cells. However, where incorporation of the plasmid DNA induced apoptosis in target cells accumulation of mutations would be prevented, as appears to be the circumstance for the $p75^{NTR}$ tumor suppressor. Indeed, the purpose of this study was to examine the role of $p75^{NTR}$ as a potential candidate for gene therapy. For the development of gene therapy methodologies, the starting point is to identify genes that, upon introduction to a cell, alter growth in such a way as to provide a potential therapeutic benefit. Through the reintroduction of $p75^{NTR}$ into xenograft tumors, we have shown, for the first time, that $p75^{NTR}$ is a potent agent that may be useful for gene therapy of tumor cells. In this context, $p75^{NTR}$ lipoplex gene therapy appears effective in reducing tumor cell proliferation and induction of apoptosis of human prostate cancer cells, the net effect of which was to reduce overall xenograft tumor volume.

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