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RESEARCH ARTICLE

Retrovirus-mediated transfer of prothymosin gene inhibits tumor growth and prolongs survival in murine bladder cancer

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To explore the potential use of prothymosin α(ProT), a putative thymic hormone, in gene therapy for bladder cancer, we generated a replication-defective recombinant retroviral vector encoding ProT and tested its antitumor effect on the MBT-2 murine bladder cancer. C_3H/HeN mice injected with MBT-2 cells in conjunction with retroviruses encoding ProT exhibited smaller tumor mass, lower tumor incidence and higher survival rate, as well as higher antitumor cytotoxic activities compared with those injected with control viruses. However, such effects were not observed in severe combined immunodeficiency mice, suggesting that ProT exerts antitumor effects through its immunomodulatory activities. Cell growth in monolayer culture and colony formation in soft agar were enhanced in ProT gene-modified MBT-2 clones, and such growth-promoting activities of ProT could be reversed if its nuclear localization signal (NLS) was deleted. To circumvent the proliferation-promoting effect of ProT on tumor cells, a retroviral vector encoding ProT lacking NLS was constructed. Our results showed that retroviruses encoding NLS-deleted ProT was more efficacious than those encoding wild-type ProT in prolonging survival of tumor-bearing mice. This is the first report indicating that ProT, in particular NLS-deleted ProT, delivered by retroviral vectors may be further explored for the treatment of bladder cancer.
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Keywords: *prothymosin α; retroviral vector; bladder tumor*

Introduction

Conventional therapy often fails in the treatment of solid tumors despite complete surgical resection. With the development of effective techniques to deliver genes into target cells, gene therapy becomes a promising alternative in the treatment of cancer. Introductions of cytokine genes with immunostimulatory or antitumor activities have shown promise in inhibiting tumor growth in animal models. Because thymic peptides, such as thymosin α1 extracted originally from calf thymus, promote the maturation of T cells, they are used in clinical trials for the treatment of patients with cancer and immunodeficiency.[1] Prothymosin α (ProT), the putative precursor of thymosin α1 that contains 113 amino acid residues with the thymosin αl sequence at its N-terminus, was originally isolated from rat thymus.[2] Many data show the behavior of ProT, like thymosin α1, as a biological response modifier. Furthermore, ProT has been reported to be more effective than thymosin α1 in the protection of mice infected with *Candida albicans*, suggesting that the biological activity of ProT is not solely determined by its N-terminal region that includes the thymosin α1 fragment.[3]

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Bladder cancer, especially presenting as superficial disease, is responsive to immunotherapeutic agents, such as BCG, and may represent a good candidate for immunological intervention. Indeed, in the murine MBT-2 bladder tumor model, irradiated, interleukin-2 or granulocyte–macrophage colony-stimulating factor gene-modified MBT-2 cells were capable of curing about half of the mice bearing wild-type MBT-2 tumors and engendered protective immunological memory in the cured mice.[4,5] Because ProT induces T cell maturation and differentiation, and possesses antitumor effects, in this study we investigated the feasibility of retrovirus-mediated ProT gene therapy for murine bladder cancer. Since ProT is implicated recently as a nuclear protein associated with cell proliferation, to circumvent the growth-promoting activity of ProT, we also constructed a retroviral vector encoding ProT deletion mutant lacking the nuclear localization signal (NLS) and examined its antitumor efficacy. Our results showed that retroviruses encoding mouse ProT suppressed MBT-2 tumor growth in syngeneic C_3H/HeN mice, but not in severe combined immunodeficient (SCID) mice, suggesting that ProT exerts antitumor effects through its immunomodulatory activities. Moreover, retroviruses encoding NLS-deleted ProT were more efficacious than those encoding wild-type ProT in prolonging survival of tumor-bearing mice. Our results reveal that retrovirus-mediated gene transfer of ProT, in particular ProT lacking NLS, may be further explored for the treatment of bladder cancer.

Results

Characterization of ProT gene-modified MBT-2 clones

To test the feasibility of ProT gene therapy of MBT-2 bladder tumor, retroviral vectors encoding ProT or NLS-deleted ProT were used to transduce MBT-2 cells *in vitro* and stable transfectants were characterized. Three ProT gene-modified MBT-2 clones, designated MBT-2/ProT 2, 10 and 14, which were chosen at random from 22 transformants were used for further characterization. Likewise, a vector control clone, MBT-2/RUF, was also used for parallel study. The expressions of the transgene in ProT gene-modified MBT-2 clones were first analyzed by RT-PCR. The *neo* signal was detectable in various MBT-2 transfectants, but not in parental MBT-2 cells (data not shown). While a small amount of endogenous ProT was present in MBT-2 cells, MBT-2/ProT clones exhibited much higher ProT expression than parental MBT-2 or MBT-2/RUF cells (data not shown). Nevertheless, the levels of ProT expression varied among these MBT-2 transfectants. A bioassay based on thymocyte proliferation for thymic hormones was used to analyze the expression of the ProT protein. The conditioned media from MBT-2/ProT clones and recombinant ProT, serving as the positive control, showed a dose-dependent enhancement of thymocyte proliferation in the presence of suboptimal concentrations of mitogen, whereas those from parental or MBT-2/RUF cells had no effect (Figure 1a). Moreover, ProT expression was highest in clone 14 and lowest in clone 2 among three independent MBT-2/ProT clones. Taken together, ProT gene-modified MBT-2 cells via retrovirus-mediated gene transfer secreted bioactive ProT protein. The proliferations of these clones in monolayer culture were determined with the [^{3}H]-thymidine incorporation assay. Figure 1b shows that thymidine incorporation was higher in all three ProT gene-transduced MBT-2 clones compared with control vector-transduced or parental MBT-2 cells, whereas no difference was found between vector-transfected control and parental cells. The ability of these MBT-2 derivatives to grow in soft agar was further analyzed. The capacity of colony formation was enhanced in all ProT gene-transduced clones, particularly in clone 10, compared with MBT-2/RUF or parental cells (Figure 1b). Collectively, these results indicate that transduction of ProT gene into MBT-2 cells resulted in an enhancement of cell proliferation in monolayer culture and colony formation in soft agar.

To measure the effect of ProT production on the tumorigenicity of MBT-2 cells, ProT-secreting MBT-2/ProT clones 10 and 14 or parental MBT-2 cells were inoculated subcutaneously (s.c.) into syngeneic C_3H/HeN mice. When C_3H/HeN mice were inoculated s.c. with 5×10^5 or 10^6 MBT-2 cells, tumor became palpable in most mice within 8 to 14 days after tumor cell implantation. Because tumor formation was never observed in mice 30 days after inoculation, mice that did not grow tumors within 30 days all achieved tumor-free survival during the observation period of 60 to 90 days. While tumor grew in all C_3H/HeN mice inoculated with 10^6 parental MBT-2 cells, injection of ProT gene-transduced MBT-2 clones 10 or 14 failed to result in tumor formation in groups of 13 and 12 mice at 30 days after injection, respectively. After 30 days, mice inoculated with wild-type MBT-2 cells began to die as tumor burden became large, whereas mice inoculated with MBT-2/ProT cells remained tumor-free at 60 days after inoculation. When mice that had been treated with MBT-2/ProT cells were challenged s.c. with parental MBT-2 cells (2×10^6) at 60 days, no tumor formation was observed in all the mice tested. These results indicate that MBT-2/ProT cells lost their tumorigenic potential *in vivo* and was able to generate immunological memory against parental MBT-2 cells.

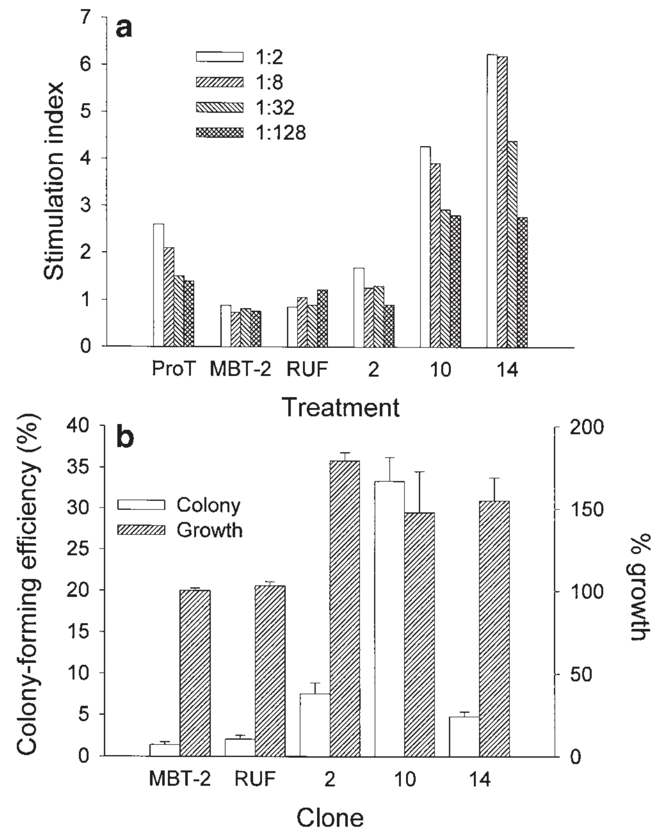

Figure 1 *The biological behaviours of ProT gene-modified MBT-2 cells. (a) The biological activities of ProT in the conditioned media of gene-modified MBT-2 clones were measured by thymocyte proliferation assay. Thymocytes (5×10^5) were incubated with four-fold dilutions (1:2 to 1:128) of the culture supernatants from various clones or with purified recombinant ProT (70 ng) in the presence of 0.125 μg/ml of Con A for 4 days. Cells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) for 16 h before harvest and cell proliferation was determined by [^{3}H]-thymidine incorporation. (b) Comparisons of colony-forming efficiency in soft agar and the growth rate among gene-modified MBT-2 clones. The colony numbers were calculated after 14 days of incubation in 0.33% soft agar. Data for colony formation is presented as colony-forming efficiency, which is the number of colony formation in soft agar divided by 5000, the original number plated. The growth rates of MBT-2 derivatives were measured by [^{3}H]-thymidine incorporation and data are presented as percentages of [^{3}H]-thymidine incorporation compared with parental cells. All data are expressed as mean ± s.e. ProT, recombinant ProT protein; MBT-2 cells, parental cells; RUF, MBT-2/RUFneo cells; 2, 10 and 14, MBT-2/RUFProT clones 2, 10 and 14, respectively.*

Induction of cytotoxic activities in mice inoculated with MBT-2 cells in conjunction with retroviruses encoding ProT

Because ProT-secreting MBT-2 cells failed to result in tumors in immunocompetent syngeneic mice, it is suggested that local secretion of ProT from the tumor cells abrogated their tumorigenicity. It is of interest to examine

whether *in vivo* administration of retroviruses expressing ProT can elicit cytotoxic activity against tumor cells. To test whether the activity of lymphokine-activated killer (LAK) cells can be enhanced in C_3H/HeN mice coinjected with MBT-2 cells and retroviruses encoding ProT, spleen and lymph node cells were isolated from treated mice at 48 days after injection and cultured in the presence of IL-2 for 4 days. The cells were then assayed for cytotoxic activity against MBT-2 using the ^{51}Cr-release assay. The results indicate that the lymphocytes of either spleens or lymph nodes from tumor-bearing mice exhibited very low cytolytic activities against MBT-2 cells. Coadministration with RUFneo control retroviruses did not show any enhancement on LAK activities. In contrast, retroviruses expressing ProT significantly enhanced the LAK activities compared with control retroviruses or PBS. The augmentation of LAK activities was more pronounced in lymphocytes from the lymph nodes than those from the spleens (Figure 2).

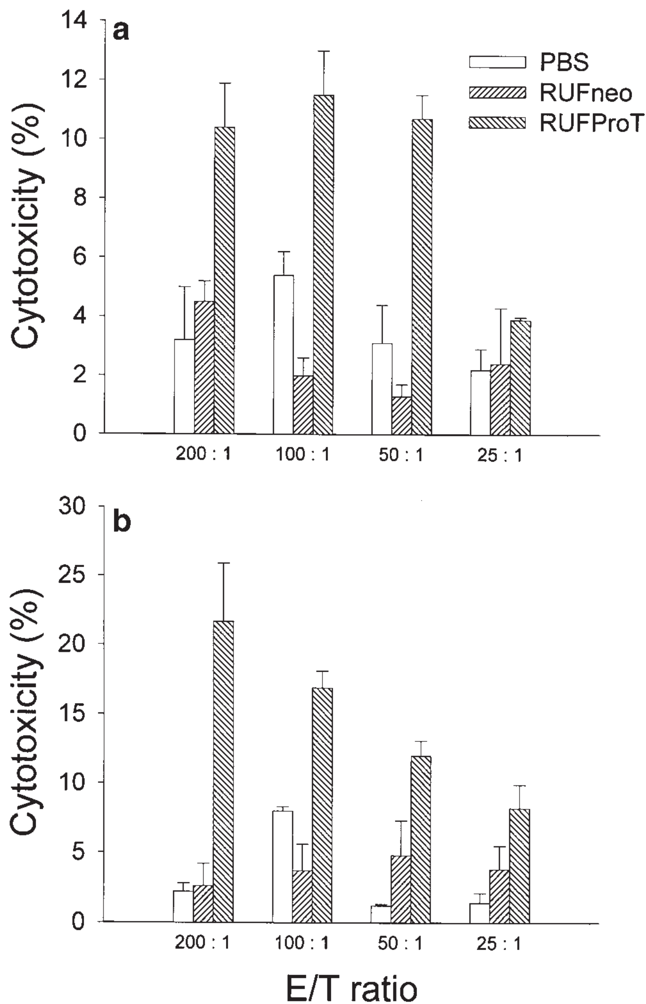

Figure 2 *Induction of LAK activity against MBT-2 cells by (a) the splenocytes, and (b) the lymphocytes from lymph nodes near the tumor site in mice coadministered with MBT-2 cells and RUFProT or RUFneo retroviruses, or PBS. C3H/HeN mice were injected with MBT-2 cells in conjunction with retroviruses or PBS. Forty-eight days later, lymphocytes from spleens and lymph nodes were assayed for cytotoxic activities against MBT-2 cells with the ^{51}Cr-release assay. E/T ratio denotes effector cell to target cell ratio.*

Effects of ProT on tumor formation of MBT-2 cells in vivo

To test the antitumor efficacy of retroviruses expressing ProT, the tumor incidence in mice inoculated s.c. with MBT-2 cells, in conjunction with recombinant retroviruses encoding ProT was compared with those coinoculated with control viruses or PBS in syngeneic C_3H/HeN mice. Tumor grew in 78% of C_3H/HeN mice inoculated s.c. with 10^6 MBT-2 cells. Coadministration with RUFneo retroviruses had no effect on tumor growth. However, those inoculated with MBT-2 cells in conjunction with RUFProT retroviruses exhibited only 30% of tumor incidence (Table 1). Moreover, as shown in Figure 3a, mice treated with RUFneo control viruses had detectable tumors at day 9 after injection and occupied larger tumor sizes compared with those treated with retroviruses encoding ProT. Throughout the experiment, tumor growth was inhibited in mice treated with RUFProT retroviruses.

To evaluate whether the immune response played a role in the antitumor effect of RUFProT retroviruses, SCID mice were used for a similar experiment. Contrary to MBT-2 syngeneic C_3H/HeN mice, tumor growth was not suppressed in SCID mice coinjected with MBT-2 cells and RUFProT retroviruses when compared with those treated with control viruses. The mean tumor volume of RUFProT-treated mice was even larger than that of RUFneo-treated mice (Figure 3b), suggesting that the antitumor effect of ProT was attributed to its immunomodulatory activities. Collectively, retroviruses encoding ProT exerted antitumor activity only in immunocompetent mice.

Effects of the NLS within ProT on cell growth

As shown in Figure 3b, the growth of larger tumors in SCID mice treated with retroviruses encoding ProT suggests that the ability of ProT to promote cell growth could be contradictory to its antitumor activity. We next examined whether the proliferation-promoting activity of ProT could be abolished if its NLS located in the C-terminus was deleted. To this end, we constructed eukaryotic plasmid vectors for expression of ProT and its NLS-deleted mutant tagged with enhanced green fluorescent protein (EGFP) in MBT-2 cells. Subcellular localization of the respective proteins was analyzed by fluorescent microscopy. EGFP-tagged ProT was localized exclusively in the nucleus, whereas EGFP-tagged ProTΔNLS distributed in both the cytoplasm and the nucleus (Figure 4a), indicating that the KKQK sequence on the C-terminus of ProT contributed to its nuclear localization.

We next generated RUFProTΔNLS retroviruses which encoded ProT lacking NLS and used the virus to transfect

Table 1 Incidence of tumor growth in C_3H/HeN mice inoculated with MBT-2 cells in conjunction with retroviruses encoding ProT

Treatment	*Tumor incidence*	*Percentage*
MBT-2	7/9	78
MBT-2 + RUFneo	8/9	89
MBT-2 + RUFProT	3/10	30

On day 0, MBT-2 cells (10^6) admixed with 1.5×10^5 CFU of retroviruses encoding ProT or neo, or with PBS were injected s.c. into groups of 7-week-old C_3H/HeN mice. The incidence rate of tumor growth at 25 days after injection is represented as: (numbers of tumor-bearing mice)/(total numbers in each group).

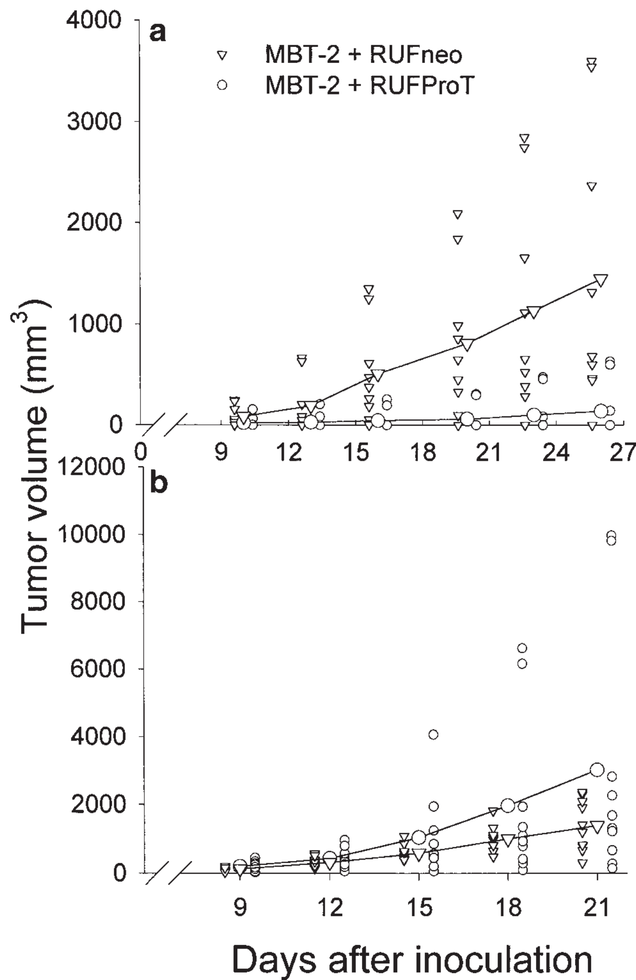

Figure 3 Effect of ProT on tumor growth in mice. MBT-2 cells (10^6) admixed with retroviruses encoding ProT or with control viruses (1.5×10^5 CFU) were inoculated s.c. into groups of nine or 10 (a) C_3H/HeN mice or (b) SCID mice. The tumor volume in each mouse is shown and the curve denotes mean tumor volume.

MBT-2 cells. Three NLS-deleted ProT gene-modified MBT-2 clones, designated MBT-2/ProTΔNLS 1, 2 and 3, were obtained for further study. The expressions of ProT in these clones were confirmed by RT-PCR (data not shown). In terms of growth in monolayer culture measured by [^{3}H]-thymidine incorporation, three MBT-2/ProTΔNLS clones proliferated more slowly than MBT-2/ProT cells (Figure 4b). Furthermore, the ability of MBT-2/ProTΔNLS clones to form colonies in soft agar was reduced compared with MBT-2/ProT cells (Figure 4b). However, the colony-forming efficiency was still slightly higher in MBT-2/ProTΔNLS clones than in control cells. To determine whether MBT-2/ProTΔNLS clones also had lower growth potential than MBT-2/ProT clones in SCID mice, groups of six SCID mice were injected s.c. with various MBT-2 clones (10^6) and tumor growth was monitored. As shown in Figure 4c, SCID mice inoculated with MBT-2/ProTΔNLS cells exhibited lower tumor size and longer survival compared with those inoculated with MBT-2/ProT cells. Furthermore, two out of three MBT-2/ProTΔNLS clones displayed similar growth potential to the MBT-2/RUF transfectant control in SCID mice. Taken together, these results demonstrate that the pro-

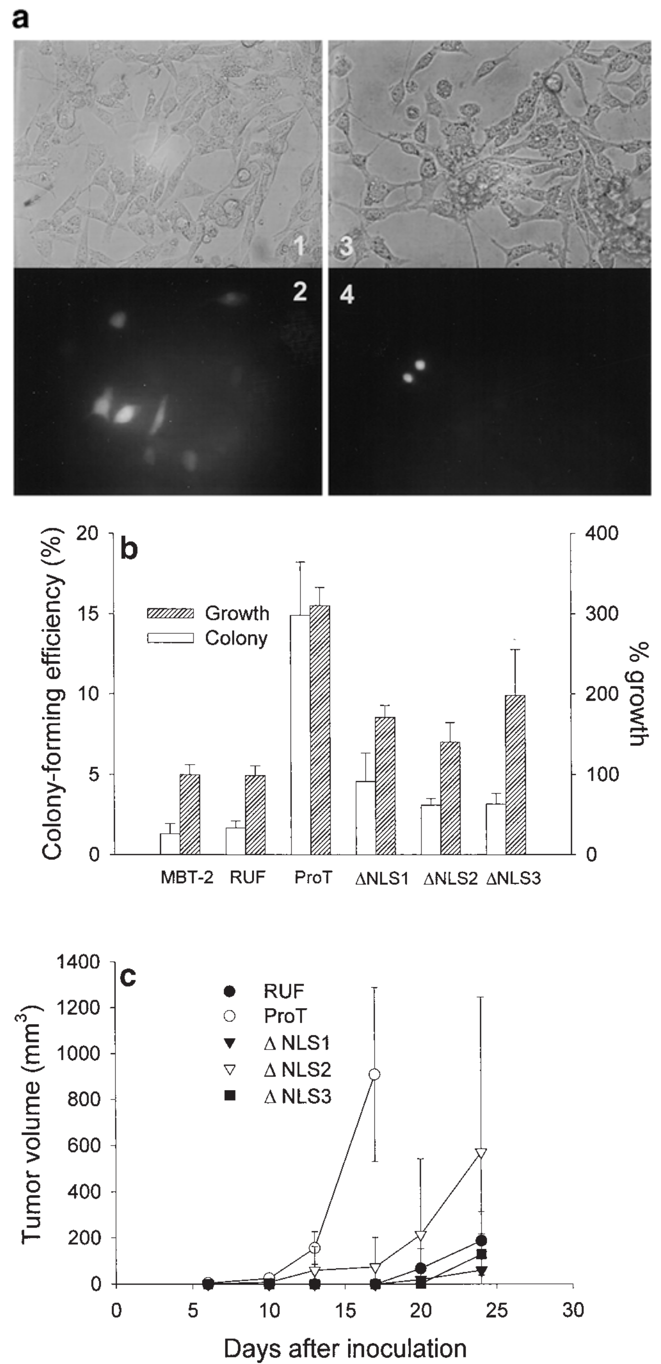

Figure 4 (a) Subcellular localization of EGFP-tagged wild-type ProT and EGFP-tagged ProT lacking NLS in MBT-2 cells. (1) Bright field micrograph of MBT-2 cells expressing EGFP-tagged ProTΔNLS and (2) fluorescent micrograph of the cells shown in 1. (3) Bright field micrograph of MBT-2 cells expressing EGFP-tagged wild-type ProT and (4) fluorescent micrograph of the cells shown in 3. (b) Growth rates of ProTΔNLS gene- or ProT gene-modified MBT-2 clones. The growth rates of MBT-2 derivatives were measured by [^{3}H]-thymidine incorporation and data are presented as percentages of [^{3}H]-thymidine incorporation compared with parental cells. Data are expressed as mean ± s.e. (c) Tumorigenicity of ProTΔNLS gene- or ProT gene-modified MBT-2 clones in SCID mice. Groups of six SCID mice were inoculated s.c. with various MBT-2 derivatives (10^6). Tumor growth and survival were monitored. The mean tumor volume in each group is presented when all mice within the same treated group were still alive. MBT-2, parental cells; vector, control vector-transfected MBT-2 cells; ProT, ProT gene-modified MBT-2 cells; ΔNLS1, ΔNLS2 and ΔNLS3, ProTΔNLS gene-modified MBT-2 clones 1, 2 and 3, respectively.

liferation in monolayer culture and colony formation in soft agar, as well as tumor formation in SCID mice were all reduced in MBT-2/ProTΔNLS clones relative to full-length ProT gene-transduced MBT-2 cells. Thus, the growth-promoting effect of ProT can be abolished when its NLS sequence is deleted.

Antitumor effects of retroviruses encoding ProTΔNLS

The final question addressed in this study was whether retroviruses encoding ProTΔNLS would exert higher antitumor activities *in vivo* compared with its wild-type counterpart. Tumor grew in 80% (12/15), 86.67% (13/15) and 100% (14/14) of mice concomitantly treated with MBT-2 cells (5×10^5) and 10^5 colony-forming units (CFU) of RUFProTΔNLS, RUFProT and RUFneo viruses, respectively. RUFProTΔNLS exhibited the lowest incidence of tumor growth compared with those concomitantly received MBT-2 cells and RUFProT or received MBT-2 cells and RUFneo, albeit the difference was not significant. In terms of tumor size, in mice inoculated with MBT-2 cells in conjunction with retroviruses encoding either ProT or ProTΔNLS, tumor growth was slower than those coinjected with control viruses (Figure 5a). Furthermore, tumor growth was significantly retarded and more than half of the mice survived for over 70 days in mice receiving RUFProTΔNLS retroviruses. The Kaplan–Meier survival curves of the three treated groups are illustrated in Figure 5b. The survival of RUFProTΔNLS-treated mice was significantly better than that of the control mice ($P = 0.0087$ by log-rank test).

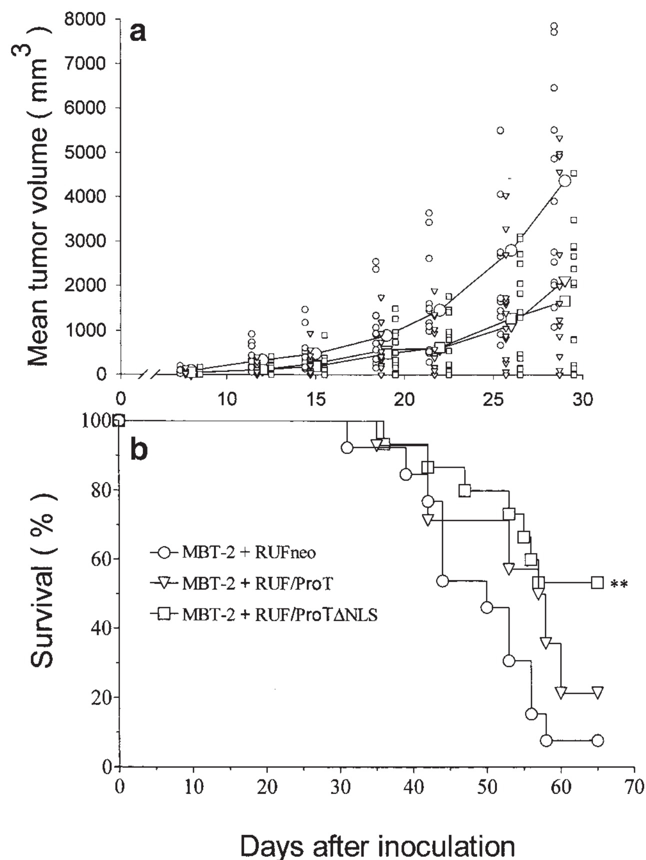

Figure 5 Antitumor effects of retroviruses encoding ProT or ProTΔNLS in the MBT-2 tumor model. Groups of 14 or 15 C_3H/HeN mice were inoculated s.c. with MBT-2 cells (5×10^5) along with 105 CFU of RUFneo, RUFProT, or RUFProTΔNLS. (a) The tumor volume in each mouse is shown and the curve denotes mean tumor volume. (b) The survival curves in each group are shown. The survival analysis was performed using the Kaplan–Meier survival curve and the log-rank test (**P = 0.0087 versus RUFneo-treated group).

Discussion

The essential role of the thymus in the development, maintenance and regulation of the immune system has been well documented. Thymic peptides have been demonstrated to be an effective immunopotentiating agent and can act in lieu on the thymus gland to reconstitute the immune function in immunosuppressed animals and in humans with a number of primary and secondary immunodeficiency. Moreover, they also show promise for boosting the immune response in patients undergoing cancer therapy. The immunomodulatory activities of ProT *in vitro* and in animal models have been described.[6] A phase II clinical trial in patients with advanced non-small-cell lung cancer showed that combined treatment with thymosin α1 and low-dose interferon-α after ifosfamide chemotherapy enhanced response rate and reduced hematologic toxicity compared with chemotherapy alone.[7] It has been shown that ProT was equally effective in stimulating the chemotactic activity of polymorphonuclear cells (PMNs) from tumor patients and healthy donors. PMNs from tumor patients, especially those derived from patients with breast tumor, were characterized by a significant enhancement of cytotoxicity against tumor target cells, as compared with healthy donors. ProT may improve some PMN functions of tumor patients, associated with the proposed role in host–tumor interaction.[8] In addition, we have demonstrated that the efficacy of DNA vaccines may be enhanced by the simultaneous expression of ProT serving as a vaccine adjuvant.[9] Collectively, thymic peptides, such as ProT, may be used as candidate genes for gene therapy in non-specific immunological enhancement of the host antitumor response.

Apart from behaving as a biological response modifier, ProT is more recently implicated in cell proliferation, as ProT gene expression occurs ubiquitously in mammalian cells and the expression of the ProT gene is correlated with cell proliferation in a wide variety of cells. We have shown previously that overexpression of ProT accelerates cell proliferation by shortening the duration of the G1 phase of cell cycle.[10] The correlation between ProT levels and cell proliferation has led to the possible application of ProT as a cell proliferation marker in human cancers, which was first described in breast cancer.[11,12] Likewise, plasma levels of its derivative thymosin α1 were proposed as a marker for the prognosis of lung cancer.[13] Thus, the levels of ProT in tumors might be employed as a prognostic factor. Furthermore, ProT has been demonstrated as a nuclear protein and the basic cluster of amino acids at its C-terminus, TKKQKT, has been identified as the nuclear targeting signal.[14] It was found that ProT is subjected to caspase-mediated fragmentation in apoptosis and this is likely to impair its intranuclear proliferation-related functioning through removal of the small fragment at the C-terminus containing the NLS.[15] Thus, it has been proposed that ProT fragmentation and relocalization in an apoptotic cell may be the first step in converting nuclear protein into an externalized bioactive

peptide with immunomodulatory activity, such as thymosin α1.[15] To this end, ProT was reported to be externalized by the cell and activate the cells of the immune system.[16] Therefore, although the physiological function of ProT remains elusive, its intracellular functions associated with proliferation and its immunomodulatory effects may not mutually exclusive. Nevertheless, if ProT is employed as a biological response modifier in an attempt to enhance the immune response in cancer patients, it would be desirable to abrogate its proliferation-promoting effect by deleting its NLS motif.

In this study, we demonstrated that ProT gene-transduced MBT-2 bladder cancer cells grew faster and formed more colonies in soft agar than their parental cells *in vitro*, suggesting that ProT may promote tumor growth, if the finding *in vitro* can be extrapolated to the *in vivo* setting. Thus, considering the Janus-faced nature of ProT towards tumor growth, we also produced retroviruses carrying NLS-deleted ProT gene and used them to generate MBT-2 transfectants expressing ProT lacking NLS. It has been demonstrated that ProT exhibits a punctuated nuclear distribution, which is related to that of transcription sites, implying that ProT is involved in transcription.[17] Our results from the distribution of the ProT-EGFP fusion protein in MBT-2 cells confirm the nuclear localization of ProT. Furthermore, we also found that the exclusive nuclear localization of ProT could be disturbed by deleting its NLS. Our finding is in accord with observations by Evstafieva *et al*,[15] who showed that while the wild-type ProT is exclusively nuclear, the C-terminally truncated ProT devoid of the NLS motif and the sequence thereafter appears to be evenly distributed within the cell. Our results showed that the growth potential of three stable MBT-2 clones expressing ProTΔNLS was reduced compared with those expressing wild-type ProT, as assessed by cell proliferation in monolayer culture and colony formation in soft agar. Furthermore, tumor growth was decreased in SCID mice inoculated with MBT-2/ProTΔNLS clones compared with those inoculated with MBT-2/ProT clones. Thus, the proliferation-promoting effect of ProT, which may contribute, in part, to tumor growth, can be circumvented by deleting its NLS sequence. In this study, the enhancement of the antitumor effects on murine bladder tumor by *in vivo* administration of retroviruses encoding ProTΔNLS compared with those carrying the wild-type ProT gene may be due, in part, to its diminished effect on growth promotion.

In the work described here, we used retroviral vectors to introduce genes encoding wild-type ProT or ProTΔNLS into MBT-2 cells, resulting in several stable transfectants. When retroviruses enter cells, the RNA genome is reverse transcribed and the DNA product becomes integrated into the host chromosomal DNA. Because the integration event is random and proviral copy numbers vary in retrovirus-transduced clones, the phenotypes among independent clones may be different. Three MBT-2/ProT clones exhibited different expression levels of ProT, with the highest expression in clone 14 and the lowest expression in clone 2. Although these clones exhibited similar proliferation potentials, clone 10 displayed much higher colony-forming capacity than clones 14 and 2. Interestingly, clones 10 and 14 did not form tumor when injected into syngeneic C_3H/HeN mice. Thus, local secretion of ProT from the genetically modified cells abrogated their tumorigenicity *in vivo*, regardless of their enhanced growth potentials *in vitro*. We also found that mice that had been treated with ProT gene-modified MBT-2 cells rejected the parental MBT-2 tumor challenge. MBT-2 is an immunogenic tumor.[18-20] It has been shown that immunization with irradiated MBT-2 cells or hypotonic membrane preparations from this tumor protected the animals against challenge with viable MBT-2 cells.[18] In previous studies, when reimplantation of 2×10^6 and 10^6 MBT-2 cells to mice at day 18, 24 h after surgical removal of the primary tumor initially implanted with 10^6 tumor cells, the incidence of rechallenged tumor formation was only 33% and 18%, respectively, suggesting that antitumor immunity existed in these tumor-free mice.[19,21] Therefore, in this study the result of tumor challenge is not unexpected if we take into account the inherently immunogenic property of MBT-2 cells. Using a nonimmunogenic tumor model would be more appropriate to evaluate the immunological memory induced by immunomodulatory molecules *per se* expressed by genetically modified tumor cells.[22]

Although the biological role of ProT has been controversial, the potential therapeutic use of ProT for patients with immunodeficiency and cancer shows promise. In the work described here, the feasibility of ProT gene therapy for cancer was tested in the murine MBT-2 bladder cancer model. ProT gene-modified MBT-2 cells secreted ProT, which exhibited biological activity of promoting thymocyte proliferation. C_3H/HeN mice inoculated with MBT-2 cells in conjunction with RUFProT retroviruses induced higher levels of LAK activities compared with those treated with MBT-2 cells, along with control viruses, and the enhanced induction of these cytotoxic activities may contribute, in part, to the antitumor responses found.

In our tumor model where *ex vivo* ProT-gene-transduced MBT-2 cells were implanted into C_3H/HeN mice, loss of tumorigenicity was more significant than using the model with *in vivo* coadministration of parental MBT-2 cells with RUFProT retroviruses. This is in agreement with the notion that the efficiency of retroviral gene transfer *in vivo* is low compared with *ex vivo* gene transfer into tumor cells. Nevertheless, we have shown previously that retroviral vectors carrying the IFN-γ gene transduced MBT-2 cells *in vivo*, which resulted in enhancing local antitumor activities.[23] We presumed that *in vivo* transduction of tumor cells with ProT gene may have occurred in mice coadministered with tumor cells and retroviral vectors carrying the ProT gene. However, the transduction efficiency and transgene expression of ProT gene transfer via retroviral vectors may vary in treated mice, which may influence the antitumor efficacy of ProT in treated mice. As a result, tumor sizes were not uniform among mice within the same treated group. For instance, tumors grew much faster in two out of 10 SCID mice treated with RUFProT retroviruses compared with the remaining mice in the same group.

The SCID mice, $Prkdc^{scid}$, are defective in the gene encoding the catalytic subunits of DNA-dependent protein kinase. Mice having homozygous deficiency of Prkdc have no detectable IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA, as well as one-tenth or less in the size of the lymphoid organs compared with their normal counterparts. Thymus, lymph nodes and splenic follicles are virtually devoid of lymphocytes and deficient in both B and T cell functions.[24] In this study, the homozygous $Prkdc^{scid}$ mice

were used in parallel with immunocompetent, syngeneic C_3H/HeN mice to investigate the antitumor effect of retroviral vectors carrying the ProT gene. Although retroviruses encoding ProT administered to C_3H/HeN mice bearing MBT-2 tumors exerted antitumor effects, such effects were not observed in SCID mice, suggesting that the antitumor activity of ProT *in vivo* could be attributable to its immunomodulatory properties. It was noted that SCID mice receiving ProT gene-modified MBT-2 cells had even larger tumor burden compared with those receiving parental MBT-2 cells. Since the growth-promoting effect induced by ProT may play a role in tumor progression, as seen in the SCID mice, it would be desirable to attenuate or abrogate this effect. Indeed, MBT-2 clones transfected with NLS-deleted ProT were less tumorigenic compared with those transfected with wild-type ProT in SCID mice. Taken together, because immunomodulatory activity of ProT is not functional in immunodeficient mice, its growth-promoting effect becomes evident. Nevertheless, this adverse effect of ProT for antitumor immunotherapy can be abrogated by deleting its NLS.

In the work described here, direct administration of retroviruses carrying the mouse ProT gene suppressed tumor growth and prolonged survival in mice with primary MBT-2 tumors. Moreover, retroviruses encoding ProTΔNLS exhibited higher antitumor activity than those encoding wild-type ProT in terms of survival. The mechanism of ProT-induced inhibition of tumor growth has not been completely elucidated, which requires further investigation. In conclusion, our results indicate the potential usefulness of ProT gene, in particular NLS-deleted ProT gene, for the treatment of bladder cancer. This is the first report to exploit ProT gene therapy as an antitumor strategy. Alterations in local appearance of thymic factors, produced by *in vivo* viral vector-mediated gene transfer, may therefore be useful in the immunotherapy of cancer.

Materials and methods

Cells and mice

The ψCRE ecotropic and ψCRIP amphotropic retroviral packaging cell lines were cultured in complete medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM L-glutamine and 50 μg/ml gentamicin.[25] MBT-2 murine bladder tumor cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 μg/ml gentamicin. Female C_3H/HeNCrj and $Prkdc^{scid}$ mice at the age of 6 to 8 weeks were obtained from the Laboratory Animal Center of the National Cheng Kung University Medical College (NCKUMC). The principles of laboratory animal care were followed and the animals were housed and fed in compliance with the operational guidelines of the Committee of Animal Facility, NCKUMC.

Generation of recombinant retroviruses

A 1.2 kb DNA fragment containing the mouse ProT cDNA was excised from the plasmid pJ6ΩProT by digestion with *Sal*I and *Bgl*II, and cloned into the *Xho*I/*Bgl*II sites of the retrovirus shuttle vector pRUFneo.[10,26] The coding region of the ProT deletion mutant lacking NLS at its C-terminus was obtained by PCR-based mutagenesis using plasmid pJ6ΩProT as the template, and two primers, 5′-ACC GAG GAG GAT TAG ACA GGA AAA GGA AAA A and 5′-GGT GTC CAC ATC GTC ATC CTC ATC ATC CTC, and subsequently subcloned into pRUFneo. The resultant plasmids pRUFProT and pRUFProTΔNLS provide eukaryotic expression elements: 5′ long terminal repeat (LTR) promoter of Moloney murine leukemia virus (MoMLV), mouse ProT gene, neomycin phosphotransferase gene (*neo*) as positive selection module, and 3′ LTR promoter.

The pRUFProT, pRUFProTΔNLS and pRUFneo plasmids were converted into corresponding retroviruses, designated RUFProT, RUFProTΔNLS and RUFneo viruses, respectively, by transfection into the packaging cell line ψCRE with the calcium phosphate precipitation method. Twenty-four hours after transfection, the cells were selected in complete medium containing 0.75 mg/ml G418. G418-resistant colonies were isolated, and virus-containing cell-free supernatant was used to infect ψCRIP cells in the presence of 8 μg/ml polybrene. Cells were isolated by G418 (0.75 mg/ml) selection, expanded to cell lines, which were designated ψCRIP/pRUFProT, ψCRIP/pRUFProTΔNLS and ψCRIP/pRUFneo cells, respectively. Confluent culture of the cells was changed with fresh medium. Following further a 24-h incubation, the virus-containing supernatants were harvested, filtered through 0.45-μm filters and used immediately for various experiments.

Determination of retrovirus titers

Retroviral titers in the supernatants collected from various retroviral packaging clones were determined by counting G418-resistant colonies of transduced NIH3T3 cells and expressed as CFU per milliliter as previously described.[23]

Generation of MBT-2 transfectants expressing ProT or ProTΔNLS and detection of transgene expression in transfected cells

MBT-2 cells were infected with RUFProT or RUFProTΔNLS retroviruses in the presence of polybrene and clonal derivatives were isolated by G418 selection (0.75 mg/ml) and expanded to independent clones. Various MBT-2 transfectants were harvested, and total cellular RNA was isolated and used for detection of transgene expression by RT-PCR as previously described.[23] The specific primers used for mouse ProT expression were 5′-CGG GAT CCA TGT CAG ACG CGG CAG TGG and 5′-CGG AAT TCC TAG TCA TCC TCC TCG GTC TT, and those used for *neo* and β-actin expression were as described.[23]

Measurement of ProT secretion

Because ProT is capable of enhancing thymocyte proliferation stimulated by suboptimal concentration of Con A,[27] we measured ProT secretion in the supernatants of ProT gene-transduced cells based on this activity. Mouse thymocytes (5×10^5) were incubated with various dilutions of culture supernatants from various clones or with purified recombinant ProT (70 ng) in the presence of 0.125 μg/ml of Con A for 4 days.[10] Cells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) for 16 h before harvest and cell proliferation was determined by [^{3}H]-thymidine incorporation. The stimulation index denotes the ratio of [^{3}H]-thymidine incorporation into cells treated with vari-

ous conditioned media to that into cells incubated with culture medium alone.

Assays of cell proliferation and colony formation in soft agar

To determine cell proliferation in independent MBT-2/ProT and MBT-2/ProTΔNLS clones, as well as in vector control or parental cells, various cells (2×10^3) were plated in hexaplicate in 96-well microtiter plates with RPMI 1640 medium supplemented with 0.2% FCS and cultured for 24 h. Cells were refed with RPMI 1640 medium supplemented with 10% FCS and cultured for 48 h. [^{3}H]-thymidine (0.5 μCi) was added to each well for an additional 6 h of culture. The cells were harvested and [^{3}H]-thymidine incorporated into DNA was counted with a liquid scintillation counter. Data are presented as percentages of [^{3}H]-thymidine incorporation compared with parental cells.

For soft agar assay, six-well plates were coated with 1 ml of 0.6% Bacto-Agar (Difco, Detroit, MI, USA) in culture medium. Various transfectants were plated in quadruplicate or hexaplicate at 5×10^3 cells/well in 0.33% Bacto-Agar in culture medium (0.9 ml). After 2 weeks, colonies were counted from each well and the average colony number was calculated for each clone. Data are presented as colony-forming efficiency which is the average number of colony formation in soft agar divided by 5000, the original cell number plated.

Generation of ProT and ProTΔNLS tagged with EGFP and transfection procedures

The vectors expressing wild-type ProT and ProTΔNLS tagged at the C-terminus with EGFP was generated by subcloning PCR-amplified products containing the sequences of wild-type ProT and ProTΔNLS into *Bgl*II–*Eco*RI sites of the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA). All constructs were confirmed by restriction mapping and DNA sequencing. MBT-2 cells were transfected with the vectors in six-well plates as described above. The localization of ProT-EGFP fusion protein in MBT-2 cells was examined under fluorescence microscopy 48 h after transfection.

Animal studies

To assess the antitumor activity of ProT gene therapy using *in vivo* retroviral gene transfer for primary tumors, C_3H/HeN mice were injected s.c. with MBT-2 cells (10^6 or 5×10^5) along with various retroviruses as described previously.[21] To determine the tumorigenicity of MBT-2/ProT and MBT-2/ProTΔNLS transfectants compared with parental or vector control cells, various cells (10^6) were inoculated s.c. into C_3H/HeN or Prkdc^{scid} mice and tumor growth and survival were monitored thereafter. Palpable tumors were measured twice a week in two perpendicular axes with a tissue caliper and tumor volumes were calculated as: (length of tumor) × (width of tumor)^2 × 0.45. The mean tumor volumes were calculated until the animals began to die, as it was only reasonable to present the data when mice within the same treated group were all alive. Once the mice began to die, only survival time was presented.

^{51}Cr release assay for cytotoxic activities

Lymphocytes isolated from spleens or lymph nodes near tumor sites from mice at 48 days after treatment were used for determination of the LAK activity. The standard ^{51}Cr-release assay was performed as previously described.[23]

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