Delivery of PTEN via a novel gene microcapsule sensitizes prostate cancer cells to irradiation

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Abstract

The tumor suppressor gene *MMAC/PTEN* located on chromosome10q23.3 has dual phosphatase activity in the phosphoinositide-3-kinase signaling pathway and inhibits Akt activation, a serine-threonine kinase, which is involved in proliferative and antiapoptotic pathways. Furthermore, *MMAC/PTEN* is frequently inactivated in a variety of tumors including prostate cancer. In this study, we generated a new type of gene transfer drug, GelaTen, which is a microsphere of cationized gelatin hydrogels incorporating PTEN plasmid DNA. Using our previously reported radiation-resistant PC3-Bcl-2 human prostate cancer cells (PTEN deleted), we examined the efficacy of GelaTen to force the expression of PTEN *in vivo* to inhibit tumor growth after intratumoral injection alone or with irradiation. Combinational therapy with GelaTen and irradiation improved both the *in vitro* and *in vivo* efficacy of growth inhibition compared with GelaTen or irradiation alone. These data show that GelaTen gene therapy, enabling radiosensitization, can potentially treat prostate cancers that have *MMAC/PTEN* gene alterations associated with radioresistance. [Mol Cancer Ther 2008; 7(7):1864–70]

Introduction

Radiotherapy is a promising, effective, and definitive treatment option for men with localized prostate cancer. Outcomes of men undergoing radiation therapy depend on various disease characteristics (i.e., serum prostate-specific antigen, clinical stage, and Gleason score; ref. 1). Specifically, >50% of men with high-risk characteristics (serum prostate-specific antigen > 20 ng/mL, >clinical T_2, and Gleason score > 7) will have a >50% chance of failing radiation or monotherapy alone (1, 2). Thus, research has concentrated on two avenues, (*a*) delivering a higher dose of radiation and (*b*) sensitizing tumors to radiation. Due to toxicity, radiation dose escalation is limited (3). Combined hormonal therapy with radiation is now a solution to improve tumor cure and control in select patients with prostate cancer, although treatment-related toxicity and a limited therapeutic window are major concerns (4). Therefore, novel radiosensitizers targeting tumor-specific molecules are strongly required for clinical application. Thus, over the past several years, our laboratory has studied various modalities to sensitize tumors to irradiation. One gene felt to confer radiation sensitivity is *MMAC/PTEN*.

The tumor suppressor gene *MMAC/PTEN*, which is located on chromosome10q23.3 (5–7), was initially identified in malignant gliomas (5, 6, 8, 9) and is frequently disrupted in a variety of tumors such as prostate cancer (10). The major function of PTEN relies on its phosphatase activity and subsequent antagonism of the phosphoinositide-3-kinase/Akt signaling pathway (11–15). PTEN is a common target for somatic alteration during the progression of prostate cancer, and loss of PTEN leads to the up-regulation of phosphorylated-Akt and Bcl-2 and contributes to poor survival and chemoresistance in prostate cancer, which in turn correlates with high grade and advanced prostate cancer (16–18). Moreover, in the case of mice, prostate-specific deletion of the PTEN leads to prostate cancer followed by progression to invasive adenocarcinoma, and subsequent metastasis with defined outcomes (19–21).

Increased expression of phosphorylated-Akt and Bcl-2 has been associated in various radioresistant cancers (22–24). We previously showed that adenoviral gene therapy of PTEN can effectively treat chemoresistant or radioresistant bladder and prostate cancers in combination with chemotherapy or radiotherapy, resulting in a decreased expression of phosphorylated-Akt and Bcl-2 (25–28). Today, the use of nonviral vectors for cancer gene therapy is an alternative. However, several problems need to be cleared, such as which nonviral vectors to use and what are their transduction efficacies. In this study, we report a new class of nonviral gene transfer drug, GelaTen, which is a microsphere of cationized gelatin hydrogels incorporating PTEN plasmid DNA that is designed for sustained release *in vivo*. Herein, we set out to examine the transduction efficacy of GelaTen in addition to examining the efficacy of tumor growth suppression of intratumoral injection of GelaTen into established subcutaneous PC3-Bcl-2 prostate cancer tumors in nude mice. Furthermore,

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we sought to determine if combination therapy with GelaTen and irradiation improved the *in vivo* efficacy of tumor growth inhibition compared with GelaTen monotherapy alone.

Materials and Methods

Cell Lines and Plasmid Vectors

Bcl-2–overexpressing prostate cancer cell line PC3-Bcl-2, which has a *PTEN* gene deletion, was kindly provided by Dr. T. McDonnell (University of Texas M. D. Anderson Cancer Center, Houston, TX) and was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, 100 mg/mL of streptomycin, 4 mmol/L of glutamine, and 400 μg/mL of G418 at 37°C in 5% CO_2 atmosphere.

The PTEN expression plasmid vector pCA14/PTEN driven by the human cytomegalovirus promoter was applied in this study, as described previously (28). The pCA17 plasmid vector containing β-galactosidase as a transgene was also used as a control DNA plasmid vector (Microbix Biosystems, Inc.).

GelaTen Preparation

Cationized gelatin hydrogels served as carriers of plasmid DNA and were prepared, as described previously (29, 30). Briefly, gelatin was cationized through the addition of ethylenediamine, followed by cross-linking through various concentrations of glutaraldehyde. To determine sustained release of gene expression by the LacZ-expressing plasmid vector pSV-LacZ, pSV-LacZ was incorporated with cationized gelatin hydrogels and was prepared with different water content formulations. The hydrogels were then implanted into the femoral muscle of mouse, and the evaluation of *LacZ* gene expression by β-galactosidase activity in muscle tissues was measured at different time points according to the manufacturer's instructions (Invitrogen). Cationized gelatin hydrogels with 97.4% water content achieved successful long-term *LacZ* gene expression *in vivo* over 20 days compared to those with other water contents (Fig. 1). For the preparation of GelaTen, cationized gelatin hydrogels with 97.4% of water content were incorporated with the pCA14/PTEN plasmid vector. Briefly, 2 mg of cationized gelatin hydrogels and 100 μg of pCA14/PTEN were mixed in 20 μL of PBS and left at 4°C overnight, followed by the addition of 80 μL of PBS. Control cationized gelatin hydrogels incorporated with pCA17 plasmid vector (CTL gelatin) was also prepared in a similar manner.

Animal Experiments

Animal experiments were done according to the guidelines of the Animal Care and Use Committee of the Nara Medical University. Six- to 8-week-old male nude mice were injected s.c. with 1×10^6 PC3-Bcl-2 cells. Tumors were measured twice weekly and their volumes calculated according to the following formula: length (L) × width (W) × height (H) × 0.5236. When a mouse's tumor reached a volume of ~ 65 mm^3, the mouse was assigned randomly to a treatment arm. A treatment cycle consisting of only a single direct intratumoral injection of plasmid at day 0 or intratumoral injection of plasmid, followed by 5 Gy of irradiation on days 5 and 15. The second treatment cycle commenced on day 31. A lead protector was used to protect non–tumor areas from irradiation therefore preventing radiation toxicity due to whole-body irradiation.

Western Blot Analysis

Western blot was done as described previously (26). Immunoblotting was done using antibodies against using a mouse antibody against human PTEN (Santa Cruz Biotechnology) and human β-actin (Santa Cruz Biotechnology), followed by horseradish peroxidase–conjugated goat anti-mouse secondary antibody (Amersham). Protein-antibody complexes were detected by ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

Immunohistochemical Analysis

Paraffin sections were deparaffinized and immunohistochemically stained with rabbit polyclonal anti-PTEN (Zymed) and rabbit polyclonal anti–phosphorylated-Akt (Ser473; Cell Signaling Technology), as described previously (26). The reactions for the antibodies were detected using HISTFINE SAB PO kit (Nichirei).

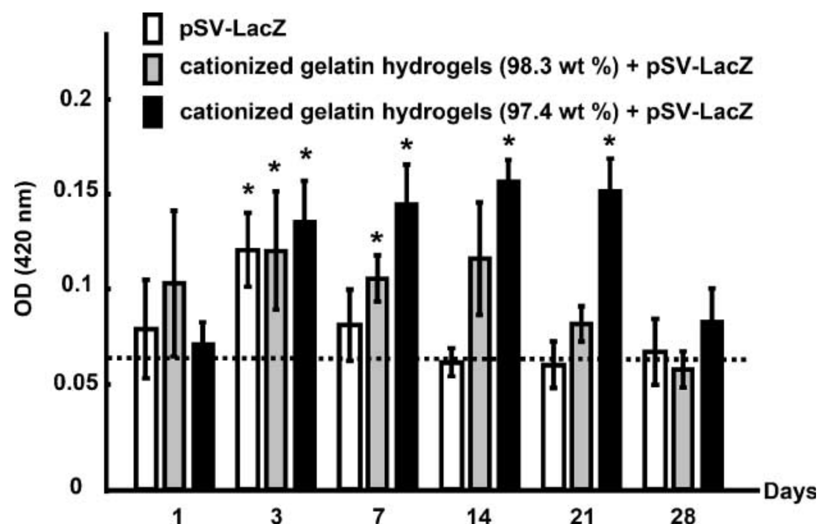

Figure 1. Optimal long-term gene expression was determined by the time course of gene expression by β-galactosidase activity, following intramuscular implantation of cationized gelatin hydrogels with pSV-LacZ. Varying water concentrations of the gelatin hydrogels were used to determine the maximal effect. *Dotted line,* the level of gene expression in the muscle of untreated normal mice (*, $P < 0.05$).

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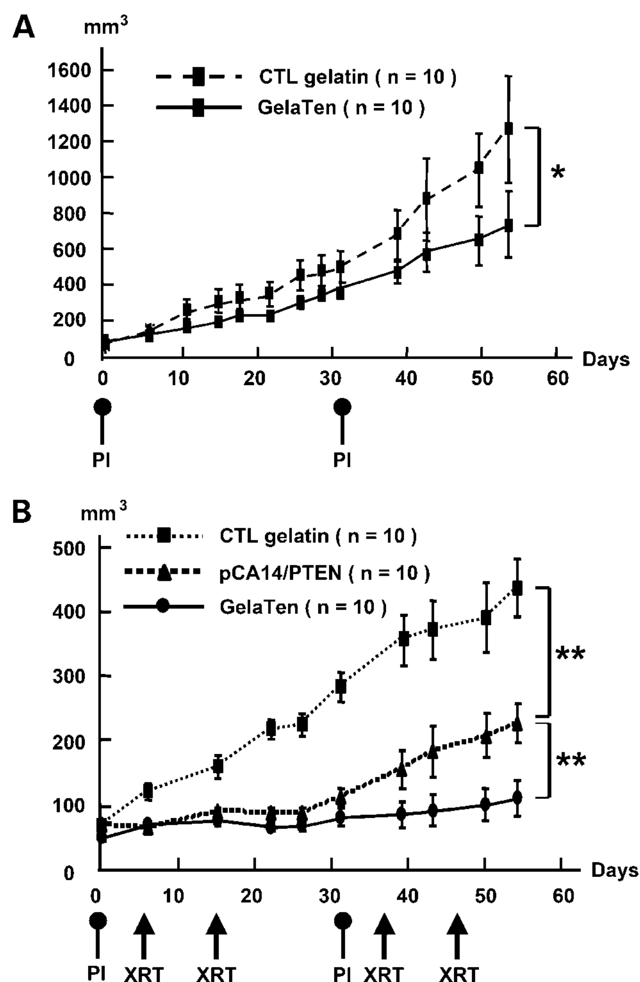

Figure 2. *In vivo* efficacy of GelaTen is represented in a 60-day time course treatment trial on PC3-Bcl-2 subcutaneous tumors by intratumoral GelaTen injection at day 0 and day 31 (**A**). Combination therapy with GelaTen followed by 5 Gy of irradiation (*XRT*) on days 5 and 15 reveals the synergistic effect induced on PC3-Bcl-2 cancer cells that have become radiosensitized by GelaTen pretreatment (**B**). Tumor volumes were measured at various intervals throughout the treatment course; *points,* mean of two independent experiments; *bars,* SD (*, $P < 0.01$; **, $P < 0.05$). *PI,* plasmid injection.

Apoptosis Detection

The extent of apoptosis in tumor samples was determined with TUNEL kit (Roche). For terminal nucleotidyl transferase–mediated nick end labeling analysis, the number of apoptotic bodies per field was counted from 10 random fields, and an apoptotic index was calculated.

Statistical Analysis

Data were statistically analyzed using Student's *t* test, and statistically significant difference was considered as $P < 0.05$.

Results

Combination Therapy with Gene Drug, GelaTen, and Radiotherapy *In vivo*

We determined the effect of PTEN on irradiation *in vitro*, the clonogenic assay of human prostate cell lines PC3 and LNCaP, which have a *PTEN* gene deletion, was done. The cell surviving fraction for both PC3 and LNCaP cells transfected with *PTEN* gene were significantly decreased (data not shown). This data suggest that exogenous expression of PTEN was the factor responsible for increased tumor sensitivity to irradiation.

To test the efficacy of GelaTen *in vivo*, we treated PC3-Bcl-2 subcutaneous tumors, which are relatively radioresistant compared with PC3 as reported previously (28), by injecting GelaTen or CTL gelatin. PC3-Bcl-2 tumors treated with GelaTen resulted in significant tumor growth suppression compared with those treated with CTL gelatin, indicated by the 649 ± 176 mm^3 versus 1,252 ± 284 mm^3 difference of tumor volume on day 54 (Fig. 2A, $P < 0.01$).

To confirm the synergistic effect of GelaTen on irradiation, we combined GelaTen tumor treatment with irradiation. Combination therapy with GelaTen and irradiation revealed significant efficacy towards tumor growth suppression compared with those treated with CTL gelatin and radiation, or pCA14/PTEN and radiation, as indicated by the 105 ± 31 mm^3 versus 420 ± 43 mm^3 and 221 ± 25 mm^3 difference of tumor volume on day 54, respectively (Fig. 2B, $P < 0.05$). Furthermore, 30% of tumors (3 out of 10 mice) treated with GelaTen and irradiation completely vanished. These data show that tumors which exhibit radiation resistance associated with overexpression of Bcl-2 can be effectively treated by administering combination therapy consisting of the gene drug GelaTen and radiotherapy.

We hypothesized that direct injection of pCA14/PTEN could introduce immediate and quick PTEN expression in tumors over a short period of time, whereas the GelaTen

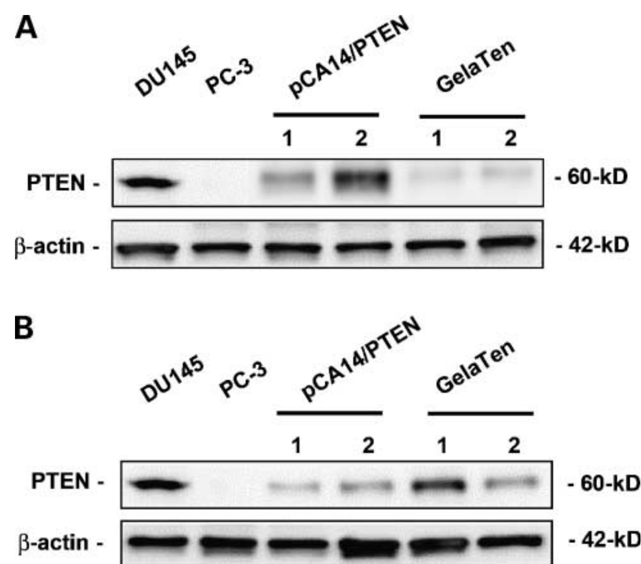

Figure 3. Western blot analysis of treated tumors harvested 10 d (**A**) and 20 d (**B**) after plasmid injection confirms PTEN expression induced by GelaTen treatment and pCA14/PTEN. Note the sustained and increased expression of PTEN by GelaTen-treated tumors over pCA14/PTEN. PC3 and DU145 cell lysates were used as PTEN-negative and PTEN-positive controls, respectively, and β-actin was used as an internal control.

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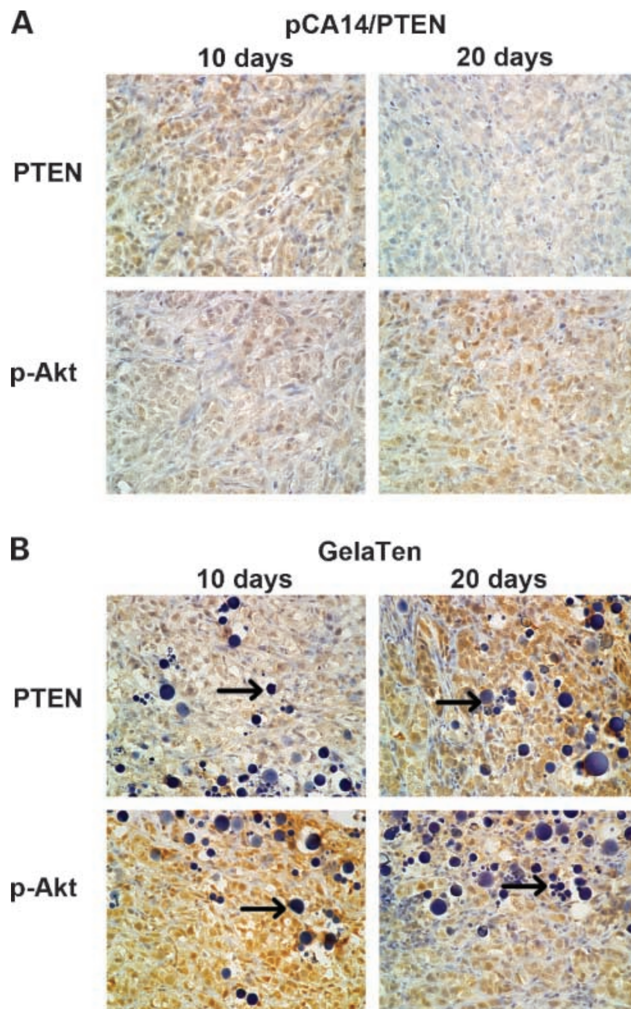

Figure 4. Immunohistochemical staining of PTEN in tumors harvested 10 d (**A**) and 20 d (**B**) after GelaTen (*arrows*) injection confirms the sustained expression of PTEN after 20 d of GelaTen treatment, thus showing its ability to slow-release the *PTEN* gene. Forced expression of PTEN results in decreased expression of phosphorylated-Akt. Magnification, ×200; *p-AKT,* phosphorylated-Akt.

treatment could induce the expression of PTEN over a longer period of time. We therefore developed an equal volume component of pCA14/PTEN and GelaTen, which was a mixture of 50 μg of pCA14/PTEN and GelaTen (1 mg of cationized gelatin and 50 μg of pCA14/PTEN) and expected a more effective treatment, that is the PTEN mixture. Tumor treatment with a direct injection of the PTEN mixture had almost the same effect as that with GelaTen (data not shown); however, the treatment with GelaTen alone was sufficiently effective. Thus, we conclude that GelaTen induces PTEN expression into tumor cells more effectively over prolonged periods of time compared with the direct pCA14/PTEN injection alone. No mice died as a result of treatments, and no significant differences in animal body weights were observed between treatment and control groups (data not shown).

Long-term Effect of Phosphorylated-Akt Inactivation by GelaTen

Using Western blot and immunohistochemical analyses, we assessed how long the injection of GelaTen or pCA/14/PTEN could induce the expression of PTEN in PC3-Bcl-2 tumors. Western blot analysis confirmed the expression of PTEN from PC3-Bcl-2 tumors treated with pCA14/PTEN or GelaTen 10 and 20 days, respectively, after plasmid injection. Western blots of tumors harvested 10 days after treatment revealed a higher expression of PTEN in PC3-Bcl-2 tumors treated with pCA14/PTEN compared with those treated with GelaTen (Fig. 3A). Conversely, tumors harvested 20 days after treatment showed markedly higher PTEN expression in the GelaTen-treated PC3-Bcl-2 tumors (Fig. 3B). Similarly, immunohistochemical analysis showed increased exogenous staining of PTEN expression in PC3-Bcl-2 tumors treated with pCA14/PTEN and GelaTen (10 and 20 days after treatment, respectively), resulting in successful down-regulation of phosphorylated-Akt in tumors treated with pCA14/PTEN (10 days) and GelaTen (20 days; Fig. 4). Because the subcutaneous tumor in nude mice is an encapsulated solid mass, it is technically difficult to administer and contain the whole GelaTen solution within the tumor proper, leading to intratumor and peritumor distribution. Thus, leakage of a certain amount of GelaTen from direct injection around the subcutaneous tumor resulted in a decreased volume of GelaTen delivered, inducing lower and delayed expression of PTEN at day 10 compared with those of skeletal muscle delivery. However, we were able to show a successfully sustained *PTEN* gene expression up to day 20 after direct injection of GelaTen.

This data shows the sustained slow-releasing feature of GelaTen to effectuate the delivery of PTEN into tumor cells, whereas also decreasing phosphorylated-Akt activation.

Apoptosis Induced by GelaTen

We did a terminal nucleotidyl transferase–mediated nick end labeling assay to determine whether GelaTen treatment can induce apoptosis in tumor cells *in vivo*. The apoptotic index was calculated in PC3-Bcl-2 tumors on day 54 (day 22 of the second treatment cycle) after treatment with GelaTen or CTL gelatin in combination with/without irradiation (Fig. 5A). In combination treatment of radiation and GelaTen, a significant increase of apoptosis on both day 42 (day 10 of the second treatment cycle) and day 54 was observed compared with that of the CTL gelatin (Fig. 5B, $P < 0.05$). In the case of the treatment group without irradiation, the apoptotic index in the tumor treated with GelaTen was significantly increased compared with the group treated with CTL gelatin, which is indicated by an apoptosis index of 0.90 ± 0.31 versus 0.30 ± 0.48, respectively ($P < 0.05$). The apoptotic index in the tumor treated with CTL gelatin and irradiation was 1.00 ± 0.47, which was not significantly different compared with GelaTen treatment alone. The apoptotic index in combination therapy with GelaTen and irradiation was 2.10 ± 0.57, demonstrating a significant increase in the number of apoptotic cells compared with GelaTen or irradiation

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treatment alone ($P < 0.05$). This shows that GelaTen has an additive effect towards the induction of apoptosis when combined with radiation.

Discussion

In this study, we showed that prostate carcinoma cells transiently transfected with pCA14/PTEN were sensitized to the killing effects of radiation *in vitro* and *in vivo*. These data indicate the importance of PTEN as one of the critical targets in the treatment of radiation-resistant prostate cancer. Moreover, we predict that PTEN is also effective in other tumors which exhibit radiation resistance associated with the overexpression of phosphorylated-Akt or Bcl-2.

Developing new approaches for gene therapy against prostate cancer is critical as there is no effective treatment for patients with advanced stages of prostate cancer. Cancer gene therapy may be defined as the transfer of recombinant DNA into human cells to achieve an antitumor effect. Such examples are shown in radiosensitization strategies in which the *HSV-tk* gene therapy aiming at the activation of a pro-drug, such as ganciclovir (31) or 5-fluorocytosine (32), can function to sensitize cells to the effects of radiation. An efficient and sustained gene induction in combination with chemotherapy or radiotherapy is critical (26, 28). Today, various viral and synthetic vectors are available, each with characteristic advantages and limitations. In choosing various vectors of gene therapy, many attributes must be considered, such as maximal transgene size permissible, maximal titer of viruses attainable, tendency to provoke inflammatory/immune response, persistence of gene expression, ability to transduce nondividing cells, effect of vector genome on desired therapy, target cell specificity, and transduction efficiency (33). Viral vectors are used in the vast majority of gene therapy trials owing to their relatively higher efficiency at gene transfers compared with the synthetic vectors; however, using viral vectors for cancer gene therapy is controversial due to toxicity and the potential for insertional mutagenesis. Several nonviral methods for gene delivery are under investigation, including chemical

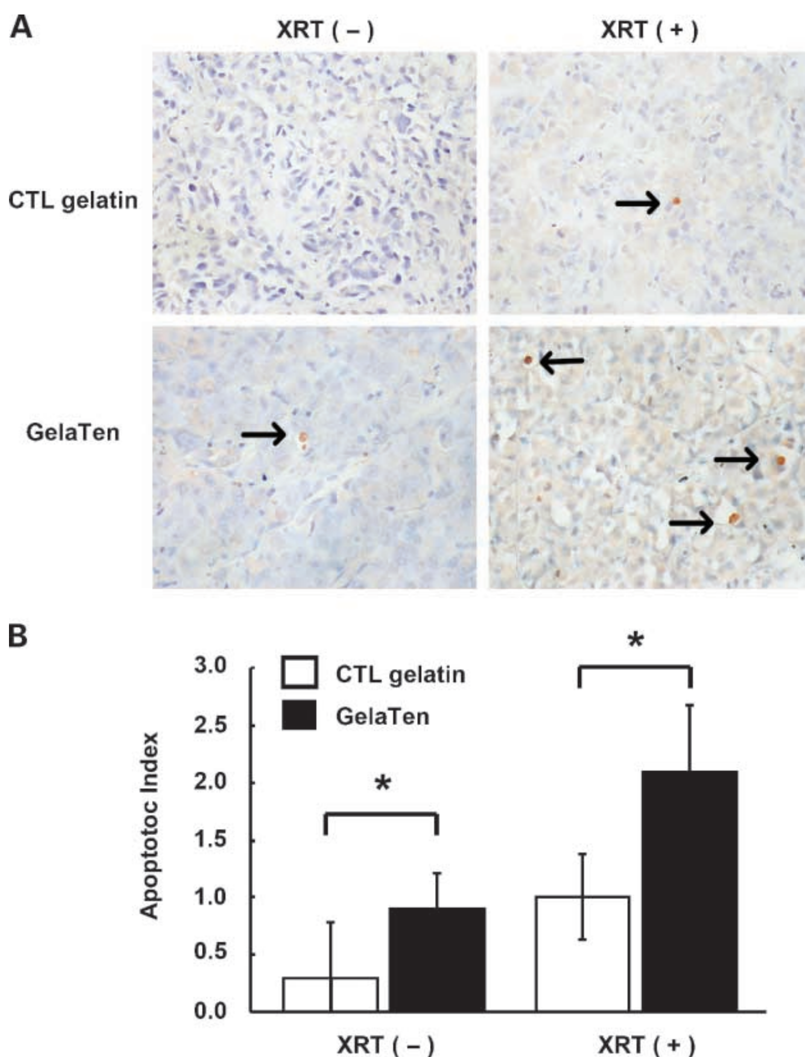

Figure 5. Combined treatment of GelaTen and radiation results in an increased induction of apoptosis *in vivo*. **A,** apoptotic cells (*arrows*) are shown by the terminal nucleotidyl transferase–mediated nick end labeling assay on PC3-Bcl-2 tumors 54 d after treatment with CTL gelatin or GelaTen combined with irradiation (magnification, ×200). The apoptotic index in the tumor treated with GelaTen was significantly increased versus that treated with CTL gelatin (**B**). The apoptotic index was based on 10 random fields scored at high magnification for treatments with [XRT(+)] or without [XRT(−)] irradiation; *columns,* mean of apoptotic bodies per field; *bars,* SD (*, $P < 0.05$).

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methods by liposomes or physical methods by microinjection, electroporation, or bioballistics (34). Nonviral methods have advantages such as being less toxic or less expensive, as well as disadvantages, such as poor transduction efficiency or shortened activity.

The simplest form of gene therapy is by using plasmid DNA. A plasmid containing a specific gene of interest and an appropriate promoter is injected directly into a desired site. However, short activity and efficient delivery of plasmid DNA are serious drawbacks *in vivo*. Gelatin has been extensively used for industrial, pharmaceutical, and medical applications, and its biosafety has been proven through its long clinical usage as a surgical biomaterial and drug ingredient (35). Generally, gelatin is not degraded by simple hydrolysis but by proteolysis. Because the plasmid vector pSV-LacZ itself is of negative charge, and gelatin is of positive charge, the pSV-LacZ molecules, once ionically complexed with the cationized gelatin, cannot be released from cationized gelatin hydrogels unless hydrogel degradation takes place to generate water-soluble gelatin fragments. It is possible that the pSV-LacZ–cationized gelatin complexes released are ionically trapped by the negatively charged substances of the extracellular matrix, resulting in its prolonged presence in the body. If the pSV-LacZ–cationized gelatin complex has a positive charge, the charge promotes the internalization of the pSV-LacZ into cells because the cell surface is negative. The controlled release by cationized gelatin hydrogels enables the pSV-LacZ not only to increase the local concentration but also to prolong the period of enhanced concentration at the implanted site of hydrogels (30).

In conclusion, we have shown how a new type of nonviral gene transfer drug, microcapsules of cationized gelatin hydrogels incorporating plasmid DNA, can be an efficacious and promising clinical tool. GelaTen is a microsphere incorporating plasmid DNA and is designed to release plasmid DNA continuously over a long period *in vivo*. Furthermore, GelaTen is not disrupted and retains its effectiveness after exposure to irradiation. Combination therapy with GelaTen and radiotherapy was more effective against tumors that exhibited radiation-resistance associated with expression of phosphorylated-Akt and Bcl-2. It has been shown that *in vivo* radiation effects persist well over a month (36), and the combination with chemotherapy has shown the same manner of increased apoptosis induction as proved in our study when combined with irradiation (37). As the result of enhanced efficacy of radiation by GelaTen in this study, a long-term sustained PTEN gene induction and expression took advantage of the radiosensitization rather than the transient *PTEN* gene expression by pCA14/PTEN plasmid alone.

This is a pilot study assessing efficacy, although we did not experience any serious adverse effects, subsequent preclinical studies will need to assess toxicity. The treatment modality using cationized gelatin hydrogels will be a novel class of nonviral gene transfer strategy and could overcome major concerns of conventional gene therapy, such as gene transduction.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were closed.

Acknowledgments

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