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GENE GUN PARTICLE ENCODING PREPROENKEPHALIN cDNA PRODUCES ANALGESIA AGAINST CAPSAICIN-INDUCED BLADDER PAIN IN RATS

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ABSTRACT

Objectives. To evaluate the efficacy of gene therapy using a gene gun or direct injection for the transfer of human preproenkephalin (PPE) plasmid cDNA using a capsaicin-induced bladder pain model in rats. Opioid peptides play an essential role in the modulation of micturition reflex and control of inflammatory pain. PPE is one such precursor molecule.

Methods. Human PPE cDNA was cloned into a modified pCMV plasmid and delivered into the bladder wall of adult female rats by direct injection or gene gun. At 4 and 7 days after gene therapy, continuous cystometrograms were performed under urethane anesthesia by filling the bladder (0.08 mL/min) with saline, followed by 15 μM capsaicin. Immunohistochemical staining was used to detect enkephalins in the bladder after PPE cDNA transfer.

Results. The intercontraction interval was decreased after intravesical instillation of capsaicin (65.0% and 63.1% decrease) in the control group or direct PPE gene injection group, respectively. However, the gene gun-treated group showed a significantly reduced response to capsaicin instillation at day 4 and day 7 (intercontraction interval 16.2% and 42.8% decrease, respectively). This analgesic effect was reversed by intravenous naloxone, an opioid antagonist (5 mg/kg). Increased enkephalin immunoreactivity in the bladder was observed in the gene gun-treated group at day 4, which was reduced at day 7.

Conclusions. The PPE gene can be effectively transferred and suppress the nociceptive response in the bladder using the gene gun method. These results support potential clinical application of PPE gene gun delivery system for the treatment of bladder pain and other types of visceral pain. UROLOGY **65:** 804–810, 2005. © 2005 Elsevier Inc.

Interstitial cystitis (IC) is a chronic inflammatory disease of the bladder characterized by bladder pain, frequency, and urgency without obvious pathologic factors. Although its etiology is poorly understood, it is hypothesized that the final common pathway of IC is activation of subepithelial afferent nerves with provoked bladder pain and a hypersensitive bladder.[1,2] Various modes of therapies for IC are available; however, current methods have had limited clinical effects on this frustrating disease.[3] Thus, it is imperative to develop new therapies for those with refractory IC and bladder pain.

Previous studies have shown that endogenous and exogenous opioid peptides can interact with receptors on sensory nerves to inhibit nociceptive responses during inflammation, as well as suppression of micturition.[4,5] Peripherally acting opioid agonists, unable to cross the blood-brain barrier and devoid of central side effects such as respiratory depression,[6] could be developed for the attenuation of nociception and treatment of painful bladder conditions. This peripheral endogenous opioid-mediated analgesia could possibly be induced by implantation of a therapeutic painkiller gene to control the inflammatory pain, such as

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bladder pain in patients with IC. The preproenkephalin (PPE) gene is such a gene, the main product of which, enkephalin, is synthesized in a variety of central and peripheral neurons and can be released from their peripheral terminals and reduce nociception by activating peripheral opioid receptors.[7–10]

The success of gene therapy is largely dependent on the development of a vector that can deliver and efficiently express a gene to the targeted tissues without obvious toxicity. The gene gun is a physical method using gold particles coated with DNA that are bombarded directly on the tissue by either electric discharge or a pressurized helium pulse.[11,12] This approach allows DNA to penetrate into the cytoplasm of the target cells and is highly effective for gene delivery.

For the present study, we hypothesized that targeted and localized expression of enkephalins by gene gun delivery of PPE cDNA could be used to treat bladder pain. Our current results showed successful transgene expression of enkephalins in the bladder and an inhibitory opioid effect on bladder overactivity induced by intravesical capsaicin in urethane-anesthetized rats.

MATERIAL AND METHODS

Human PPE cDNA Construction

Total RNA of a human adrenal gland tumor was obtained by TriZol Reagent (Clontech, Palo Alto, Calif). The cDNA was synthesized by reverse transcriptase using an oligo-dT as primer (BcaBEST RNA PCR kit, TaKaRa Biomedicals, Otsu, Shiga, Japan). The PPE gene was then amplified by polymerase chain reaction with specific upstream (5′-GGC CTG CTC CAT CCG AAC AGC GTC-3′) and downstream (5′-GCC ACT AGT GGG AAA AGA TAT-3′) oligoneucleotides. The polymerase chain reaction product contained the full-length PPE coding cDNA and was the expected 662 bp in length.[13] The amplified fragment was cloned into a pUC18 vector, and white colonies (LacZ mutation) were selected. The plasmid DNA was purified using the Mini Plasmid DNA Preparation Kit (Qiagene, Valencia, Calif), digested with restriction enzymes and electrophoresed on agarose gel. Plasmids with the correct insert were further verified by DNA sequencing, after which the PPE cDNA was digested with KpnI and inserted into the pCMV-script vector (Stratagene, La Jolla, Calif).

DNA Gold Particle Preparation and Gene Gun Injection

Plasmid DNA was precipitated onto 1.5 to 2.0-μm gold particles. In this experiment, 35-mg gold particles and 105-μg plasmid DNA were used. We used the versatile Helios Gene Gun System (BioRad, Hercules, Calif), which allows rapid and direct transfer of DNA-coated gold microcarriers into a range of targets in vivo.[11] Each pulse of helium expels the DNA-coated gold beads from a single 0.5-in. segment of gold bead-coated tubing and results in a delivery of 0.5 mg of gold and 1.5 μg of plasmid DNA per pulse.

Animal Procedures

All experimental procedures were performed on female Sprague-Dawley rats (weight 270 to 320 g). The Institutional Animal Care and Use Committee reviewed and approved all experimental procedures before the study began. Under halothane anesthesia, three types of experiments were performed after a low midline incision to expose the bladder: (a) for sham operation, the animals were bombarded with bullets with helium only (n = 6); (b) direct injection of 10 μg of plasmid PPE cDNA in a total volume of 0.1 mL saline using a 30-gauge needle into four sites of the bladder wall (n = 6); and (c) four bullets of PPE cDNA particle bombarded at four sites of the bladder wall (n = 12).

Cystometrography

At 4 or 7 days after gene therapy or sham treatment, the rats were anesthetized by subcutaneous injection of urethane (1.2 g/kg). A PE-50 tube (Clay-Adams, Parsippany, NJ) was inserted into the bladder through the urethra and connected by a three-way stopcock to a pressure transducer and syringe pump to record the intravesical pressure and infuse solutions into the bladder, respectively. A control cystometrogram (CMG) was performed by filling the bladder with saline (0.08 mL/min) to elicit repetitive voiding. The amplitude, pressure threshold, pressure baseline, and intercontraction interval (ICI) of reflex bladder contractions were recorded.[2] The measurements for each animal represent the average of 3 to 5 bladder contractions.

After a baseline measurement was established with saline infusion, we instilled capsaicin (15 μM) into the bladder at 0.08 mL/min for at least 30 minutes to promote acute bladder overactivity. The rats were then administered naloxone hydrochloride (5 mg/kg intravenously), an opioid receptor antagonist, to determine whether the effects of PPE gene gun treatment were mediated by opioid receptors.

Immunohistochemistry

At the end of the CMG, the animals were killed, and the bladder tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline for 4 hours and then in 30% sucrose in phosphate-buffered saline overnight for histologic examination. The samples were cryosectioned to a thickness of 10 μm and blocked in 2% normal goat serum for 2 hours at room temperature. The sections were then incubated with rabbit anti-met-enkephalin antibody (1:500, Chemicon, Temecula, Calif) in 10% normal goat serum and 0.3% Triton X-100 (Sigma, St. Louis, Mo) for 7 days at 4°C. To ensure that immunostaining was specific, control samples were incubated in the same solutions without the primary antibody. The sections were washed four times with 0.2% Triton X-100 in phosphate-buffered saline, and then incubated with fluorescence-conjugated secondary antibodies (a mixture of fluorescein isothiocyanate, Molecular Probes, Eugene, Ore) for 1 hour. The slides were then sealed using the Prolong Antifade Kit (Molecular Probes) and subjected to image analysis. To record the images, the slides were examined with a fluorescence microscope (Leica, Wetzlar, Germany). The histologic findings using hematoxylin-eosin staining were assessed by an observer unaware of the experimental findings.

Statistical Analysis

The quantitative data are expressed as the mean ± standard error. Statistical analyses were performed using two-way analysis of variance with Bonferroni post-tests as applicable, with $P < 0.05$ considered significant.

RESULTS

Bladder Activity in Animals with Direct PPE Gene Injection

As shown in Table I, the CMG parameters during intravesical saline and capsaicin instillation were not significantly different between the sham operation group and direct plasmid PPE gene injection group. These results indicate that bladder function under normal or hyperactive conditions was not affected by direct PPE gene injection. During continuous capsaicin infusion, rats were treated with intravenous naloxone hydrochloride. No change was seen after naloxone administration, consistent with the inability of the direct gene injection to alter the pain response.

Bladder Activity in Animals with PPE Gene Gun Treatment

The CMG parameters during saline-infused control CMG were not significantly different between the animals treated with gene gun PPE cDNA and sham operation (Table I). These results indicate that normal bladder function was not affected by PPE gene gun treatment.

In the sham-operation rats, the ICI was significantly reduced by 65.0% (from 11.7 ± 1.1 to 4.1 ± 0.5 minutes); however, the reduction of the ICI induced by capsaicin in the PPE gene gun injection rats was significantly smaller at day 4 (ICI reduction of 16.2%, from 9.9 ± 1.6 to 8.3 ± 1.5 minutes; Table I and Fig. 1). The antinociceptive effect of PPE gene gun injection at day 7 was also significant, but less intense than at day 4 (ICI reduction of 42.8%, from 9.8 ± 0.7 to 5.6 ± 0.6 minutes). These results indicate that the therapeutic effect of PPE gene gun injection on bladder pain could persist at least for 7 days.

During continued capsaicin infusion, administration of naloxone hydrochloride significantly antagonized the antinociceptive effect of PPE gene injection by reducing the ICI from 8.3 ± 1.5 minutes to 5.4 ± 1.2 minutes ($P < 0.05$) at day 4 or from 5.6 ± 0.6 minutes to 3.9 ± 1.1 minutes ($P < 0.05$) at day 7 (Table I and Fig. 1). These results imply that the antinociceptive response to the intravesical administration of capsaicin in the PPE gene gun-treated rats was mediated through the opioid receptors.

Histologic and Immunohistochemistry Findings of Enkephalin in PPE Gene Gun Injection Bladder

Injection of PPE cDNA by the gene gun did not cause any gross histologic changes to the bladder, as determined by the histopathologic evaluation of tissue sections stained with hematoxylin-eosin (Fig. 2). Enkephalin production in PPE gene gun-treated bladder tissues was confirmed by immuno-

TABLE I. *Effects of saline, capsaicin, and naloxone on CMG parameters in sham operation group, plasmid PPE gene injection group, and PPE gene gun-treated group on days 4 and 7 (n = 6 in each group)*

Parameter	Sham (A)			Plasmid (B)			Gene Gun Day 4 (C)			Gene Gun Day 7 (D)		
	Sal	Cap	Nal	Sal	Cap	Nal	Sal	Cap	Nal	Sal	Cap	Nal
ICI (min)	11.7 ± 1.1	4.1 ± 0.5	5.7 ± 0.7	10.3 ± 2.1	3.8 ± 1.1	4.4 ± 1.2	9.9 ± 1.6	8.3 ± 1.5	5.4 ± 1.2	9.8 ± 0.7	5.6 ± 0.6	3.9 ± 1.1
PB (cm H_2O)	7.2 ± 0.7	6.8 ± 0.6	7.0 ± 0.5	6.3 ± 1.0	7.5 ± 0.2	6.7 ± 0.5	8.8 ± 1.4	7.7 ± 1.2	7.3 ± 1.2	7.7 ± 0.5	7.8 ± 0.4	7.3 ± 0.5
PT (cm H_2O)	8.9 ± 1.2	8.4 ± 0.9	8.4 ± 0.5	13.0 ± 3.3	10.8 ± 0.8	11.5 ± 2.2	14.1 ± 1.4	11.0 ± 1.4	9.0 ± 1.2	15.8 ± 2.7	10.0 ± 0.8	8.7 ± 0.5
Amp (cm H_2O)	31.7 ± 1.4	32.5 ± 2.6	29.0 ± 1.4	40.0 ± 5.9	44.9 ± 9.2	39.6 ± 1.6	23.6 ± 1.7	26.1 ± 2.9	23.5 ± 3.7	25.7 ± 2.3	22.7 ± 2.7	19.8 ± 2.8

Data presented as mean ± SE.
ICI after Cap treatment: A vs. B, A vs. D, and B vs. D, P = 1.0; A vs. C, P = 0.042 (significant); B vs. C, P = 0.025 (significant); C vs. D, P = 0.383 (Bonferroni post-test).
Key: CMG = cystometrography; PPE = preproenkephalin; Sal = saline; Cap = capsaicin (15 μM); Nal = naloxone (5 mg/kg intravenous); ICI = intercontraction interval; PB = pressure baseline; PT = pressure threshold; Amp = amplitude.

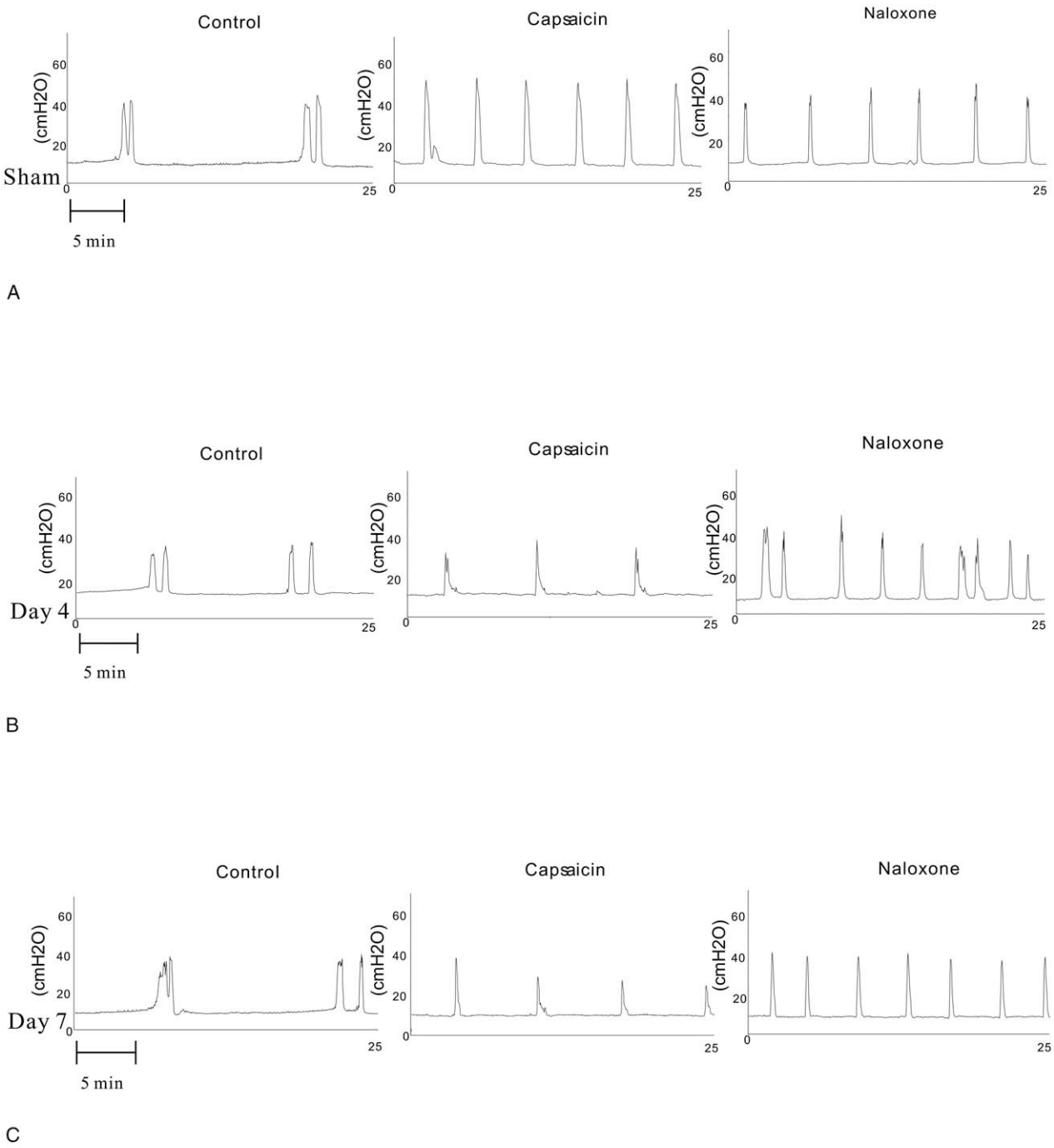

FIGURE 1. *Representative traces of in vivo continuous CMGs in urethane-anesthetized rats. CMG of (A) sham operation rat and (B) rats at day 4 and (C) day 7 after PPE gene gun injection. After capsaicin infusion, PPE gene gun-treated rat and sham-operation rat had a reduction in ICI; however, the reduction in ICI was significantly smaller (antinociceptive effect) in PPE gene gun-treated rat. Naloxone hydrochloride (5 mg/kg intravenous) reversed this effect.*

histochemical analysis, in which sections of bladder transfected with PPE cDNA particles were positive for enkephalin immunoreactivity (Fig. 2). Areas of gene expression were distributed throughout the bombarded area, with the most intense staining in the seromuscular layers, and decreasing with depth, and to a less extent, the mucosal layers. The intensity of gene expression was stronger at day 4 than at day 7.

COMMENT

Although a standard model for visceral pain has not been established, exposure of the blad-

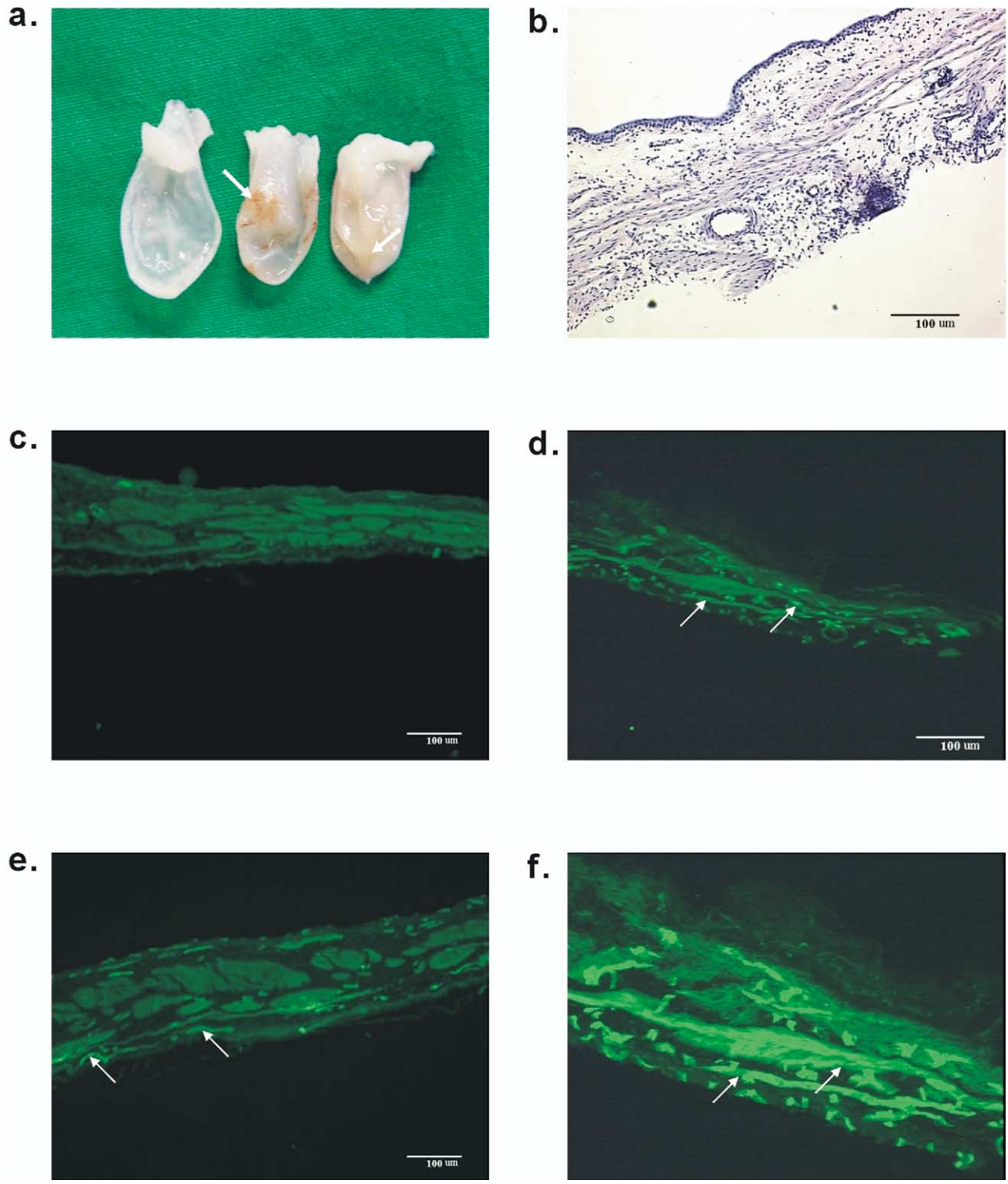

FIGURE 2. *Rat bladders showing gold particles (arrows) injected by gene gun. Gene gun uses 400-lb/in^2 helium pulse to accelerate plasmid-coated gold particles through cell membranes of urinary bladder. (a) Left, sham operation; middle, day 4; and right, day 7 after gene gun treatment. Photomicrographs of bladder sections after PPE gene gun treatment showing (b) no significant inflammatory cell infiltration on hematoxylin-eosin staining, (c) immunohistochemical detection of met-enkephalins in the sham-operated rat, and in the PPE-treated rat at (d) day 4 and (e) day 7. Original magnification ×100. Bar: 100 μm. (f) Higher magnification of part D. Original magnification ×200.*

der mucosa in the rat to capsaicin, a C-fiber stimulant, has been widely used in the study of visceral pain.[14–16] Intravesical capsaicin administration in animals resulted in an increased frequency of micturition and intense grooming or licking of the ventral abdomen or periurethral

region, indicative of bladder nociceptive responses.[15,17]

The results of a previous study have suggested that among the various opioid receptors, delta-opioid receptors may be present on the nociceptive afferents of the bladder and may be amenable to activation for production of peripheral analgesia.[8] Thus, enkephalins, which interact with delta-opioid receptors, could be a principal physiologic target for the clinically important opioid analgesics. The rationale for using the PPE gene is based on the role of enkephalin as a potent native opiate. Gene therapy with PPE cDNA targeted at the bladder may secrete enkephalins, which bind to the receptors on the peripheral terminals of primary afferents, inhibit release of inflammatory mediators, and counteract bladder pain. Because the local expression of enkephalin was detected in the bladder wall after bladder PPE gene gun treatment in this study, the antinociceptive effects of gene gun PPE gene transfer are likely to be induced by the local mechanism mediated by bladder enkephalin expression.

In vivo gene transfer is achieved by two classes of vectors: viral and nonviral. Recent safety concerns regarding viral vectors have led to renewed interests in developing nonviral vectors that can safely and efficiently express the therapeutic gene. Direct injection of naked plasmid DNA is the simplest way for DNA delivery and has been reported to transfect in skeletal muscle, liver, thyroid, brain, and urologic organs.[18] However, we could not detect any PPE effect by this method. Naked DNA injection has some disadvantages, for example, a low efficiency of transfection, a limited distribution to adjacent tracts of injection, and rapid degradation by nuclease and the mononuclear phagocyte system.[18,19] In contrast, gene therapy using the gene gun method has the advantage of safety, simplicity, and a high level of gene transfection without the risk of viral manipulation.[11,18,20] Here, we demonstrated successful implementation of gene gun for delivery of PPE gene into the bladder without apparent toxicity and side effects. Gene expression was seen to be high level, deep penetration, and maintained for at least 7 days. This concept of PPE gene transfer may be applicable for painful bladder syndromes in which IC is a dominant component.

However, the major drawback of the gene gun method is the relative short duration of transgene expression. As pointed out by Ledley,[21] in in vivo nonviral systems, DNA is gradually eliminated from the target cell, leading to a commensurate decrease in the level of expression of the gene product over time. Thus, nonviral gene therapy provides a finite period of expression of the therapeutic product. Therefore, several clinical conditions such as infection and inflammation would benefit best from transient transfection by gene gun methods,[19,20] and chronic bladder pain conditions such as IC may require multiple administration of plasmid DNA by gene gun. In addition, the possibility of prolonged gene expression in the skin has been reported using a new gene gun design.[20] Thus, future developments of new gene gun techniques might overcome the short duration of transgene expression.

Another drawback of the currently available gene guns is the requirement for surgical exposure of the targeted tissue. The development of a system that can deliver DNA to internal organs without major surgery would greatly add to the potential uses of gene gun technology. In addition, the bladder is a specific organ, the manipulation of which by cystoscopy or laparoscopy is a minimally invasive and routinely clinical procedure for urologists. The combination of advanced gene gun therapy and modern endourology would make the bladder an attractive target organ for gene therapy.

The suppression of capsaicin-induced bladder overactivity in PPE gene gun-injected rats was reversed by naloxone hydrochloride (5 mg/kg). A previous study of our group used gene gun particles encoding pro-opiomelanocortin cDNA, which is a precursor of beta-endorphin and interacts with mu-opioid receptors to reduce bladder pain in rats.[22] The analgesic effect of pro-opiomelanocortin was reversed by lower dose naloxone (1 mg/kg). This result might imply that naloxone has a greater affinity for mu-opioid receptors than delta-opioid receptors.

CONCLUSIONS

The results of the current study suggest that the PPE gene can be transferred in the bladder using a gene gun, and increased expression of enkephalins in bladder can suppress nociceptive responses. This result demonstrated proof of the concept of a PPE gene gun delivery system for the treatment of IC and visceral bladder pain conditions if prolonged gene expression can be maintained by repeated gene gun treatments or the development of new technology for gene gun approach.

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