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Intravesical Immune Suppression by Liposomal Tacrolimus in Cyclophosphamide-Induced Inflammatory Cystitis

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Aims: Potent immunosuppressive effect of tacrolimus has encouraged its topical application for achieving local anti-inflammatory effect. However, its poor aqueous solubility presents challenges in formulating biocompatible instillations to justify the investigation of liposomes as vehicle for tacrolimus. **Methods:** Adult female Sprague-Dawley rats (N = 52) divided into 4 groups were injected with cyclophosphamide (CYP) (200 mg/kg, ip) except for sham (saline injection, ip). Other three groups were instilled with either saline (1 cc, retained for 1 hr), liposome (LP- 1 cc) or liposomal encapsulated tacrolimus (LFK- 0.2 mg tacrolimus/1 ml LP). Baseline cystometrogram was performed on day 1 and day 3 prior to bladder harvest for histological staining (N = 24) in all groups except sham. In addition, 4-hr baseline urine on day 1 and day 3 was collected from all groups for urine PGE2 assay and bladder harvested for PGE2 and IL2 assay on day 3 (N = 28). **Results:** Rats treated with LFK demonstrated suppression of CYP induced inflammatory reaction with reduced EP4 staining and bladder overactivity (intercontraction interval 61.0% decrease in untreated animals) as well as normalized the several fold elevation of IL 2 and PGE2 levels in tissue and urine. CYP induced effects were not suppressed in rats left untreated with tacrolimus. **Conclusions:** This is the first report of immunesuppression in bladder by intravesical delivery of tacrolimus using liposomes. LFK significantly inhibited CYP induced inflammatory cystitis through the modulation of IL2, PGE2, and EP4 function. These findings support investigation of local tacrolimus in cases of inflammatory cystitis refractory to conventional therapy. *Neurourol. Urodyn.* © 2010 Wiley-Liss, Inc.

Key words: bladder; cystitis; liposome; tacrolimus

INTRODUCTION

Cyclophosphamide (CYP) is an oxazaphosphorine DNA alkylating agent, used clinically for its efficacy as potent anti-neoplastic and immunosuppressant. It is used in over 200,000 patients a year to treat neoplastic, immune-mediated, and transplant-related diseases.[1] The occurrence of sterile, hemorrhagic cystitis (SHC) is the most common therapy-limiting side effect of CYP with 4% mortality from uncontrolled hemorrhage.[2]

Studies done in the past has confirmed that acrolein, a phase I metabolic product of CYP, is responsible for the edema, ulceration, hemorrhage associated with SHC caused by direct contact of acrolein with bladder lumen.[3] Recent studies have highlighted the overexpression of genes related to immune and inflammatory responses including pivotal immune cells in urothelium such as activation of CD^{4+} T-helper type 1-related chemokines after CYP injection.[4] Urinary chemokine profile of rats with inflammatory and hemorrhagic cystitis reported that changes in cytokine milieu of the bladder can activate a pro-inflammatory phenotype in the bladder through rapid infiltration of innate immune cells and kinetics of cytokines/chemokines secretion into urine correlate with the abnormal voiding and histology of rats.[5,6] These findings suggest involvement of neuroimmune interaction in SHC.

Tacrolimus (FK506) is a potent hydrophobic immunosuppressive agent that acts on inhibition of IL-2-dependent T-cell activation and has a direct inhibitory effect on cell-mediated immunity.[7] Local treatment of tacrolimus has been shown to be beneficial in an ointment or lotion formulation against inflammatory skin conditions without systemic side effects.[8] One of the key benefits from topical therapy of potent immunosuppressive drugs is that effect is naturally restricted to local immune response mechanisms and severe side effects seen on systemic therapy are limited.

We hypothesized that intravesical immune suppression achieved by local delivery of potent immune suppressants can modulate the immune response triggered in the urothelium and detrusor wall during SHC. However, the lipophilic nature of tacrolimus make it poorly water soluble,[9] which creates formulation challenges for intravesical administration and its high molecular weight can adversely influence its uptake across urothelium. In the present study, we investigated whether these problems can be circumvented by encapsulation of tacrolimus in liposomes (LPs), which are spherical vesicles and able to carry effective payload of encapsulated lipophilic drugs.[10]

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MATERIALS AND METHODS

All experimental procedures were performed on female Sprague–Dawley rats (220–280 g; N = 52) and reviewed and approved by the Institutional Animal Care and Use Committee before the study began. There were two experiments and four groups of study between each experiment, sham-received saline injection (i.p.), control-received intravesical saline + CYP (i.p.) injection, LP-received intravesical LP + CYP, LFK (tacrolimus encapsulated in LP)-received intravesical LFK + CYP. In experiment 1 (N = 24), we performed cystometrogram (CMG), and histological staining (H&E, EP4); in experiment 2 (N = 28), we performed urine PGE2 and bladder PGE2 and IL-2 assay.

Urine Collection

Baseline urine samples were obtained 4 hr on day 1 before CYP or saline injection on day 3. Urine specimens obtained from rats kept in metabolic cages were frozen immediately in liquid nitrogen and stored at −80°C before analysis.

Cyclophosphamide (CYP) Injection

On day 1, SHC was induced by intraperitoneal injection of CYP (200 mg/kg; i.p.), which is metabolized to acrolein, an irritant eliminated in the urine.[11] CYP injection was performed after completion of intravesical therapy with LP or tacrolimus encapsulated in LP.

Cystometrogram (CMG)

Animals were anesthetized by subcutaneous injection of urethane (1.2 g/kg) and kept anesthetized till day 3 with additional urethane (20–30% of the initial dose) as required and periodic hydration using subcutaneous 5% glucose saline injection. On days 1 and 3, PE-50 tubing was inserted into the bladder through the urethra and connected via a three-way stopcock to a pressure transducer and to a syringe pump for recording intravesical pressure and for infusing solutions into the bladder, respectively. CMG was performed by filling the bladder with saline (0.08 ml/min) to elicit repetitive voiding. Day 1 CMG was performed before any treatment and served as control. The amplitude (the peak pressure minus the basal pressure during each contraction period), pressure threshold (PT, the pressure immediately before the reflex contraction), pressure baseline (PB, the pressure immediately after the reflex contraction), and intercontraction interval (ICI), the average time between contractions of reflex bladder contractions were recorded. Measurements in each animal represented the average of 3–5 bladder contractions.

Preparation of Liposomes (LPs) and Tacrolimus Encapsulated in LP (LFK)

A 5 mg/ml stock solution of tacrolimus was made in methanol. LP (1.8 mg, Lipella Pharmaceuticals, Inc., Pittsburgh, PA) dispersed in physiological saline (1 ml), where the dispersion is in liposomal form. LP encapsulating tacrolimus (referred to as LFK) was prepared by loading dried film of 0.2 mg of tacrolimus (Sigma, Saint Louis, MO) into LPs dispersion. In brief, stock methanolic solution of tacrolimus (80 μl) was dried out by nitrogen in a glass tube to remove methanol. After the tube is dry, with tacrolimus sticking to inside wall of tubes, 3.6 mg of lyophilized lipid powder (LP) was added to the tube. Next we add 2 ml of deionized water to the tube and vortex vigorously for 15 min. The tube was then frozen at −20°C for 1 hr and vortexed again to achieve turbid LP suspension.

Instillation of Drugs

On day 1, after baseline CMG measurements, the intravesical catheter was tied in place by a ligature around the urethral orifice under isoflurane anesthesia. The bladder was emptied of urine and filled with 1 ml of saline, LP, or LFK for 1 hr through the catheter.

Transcardiac Perfusion

On day 3, after the CMG study, animals were deeply anesthetized with pentobarbital and sacrificed via transcardiac perfusion, first with Krebs buffer followed by 4% paraformaldehyde fixative. The animals were then dissected to harvest the bladder.

Histology and Immunohistochemistry

The bladder was fixed in 10% buffered formaldehyde for 24–48 hr, and then embedded in paraffin. The bladder tissue for histology was cut in 3 μm thick pieces and stained with hematoxylin and eosin. The inflammatory reaction (inflammatory scoring and edema scoring) of CYP-induced cystitis was graded by a score of 0–3 as follows: 0, no evidence of inflammatory infiltration or interstitial edema; 1, mild (few inflammatory cell infiltrates and little or no interstitial edema); 2, moderate (infiltration of a moderate amount of inflammatory cell infiltrates and moderate interstitial edema); 3, severe (diffuse presence of a large amount of inflammatory cell infiltrates and severe interstitial edema).[11,12]

Previous studies have demonstrated that acute or chronic CYP injection induced upregulation of EP4 receptors in bladder for PGE2, which was reduced by effective treatment.[11,13] Therefore, to evaluate EP4 immunoreactivity, bladder tissue sections were cut into 3 μm sections, dewaxed in xylene, and rehydrated to distilled water through decreasing concentrations of alcohols. Antigen retrieval was achieved by pressure-cooking tissue sections immersed in 10 mM citrate buffer at pH 6.0. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Tissue sections were then incubated with rabbit anti-EP4 receptor polyclonal antibody (Cayman Chemical, Ann Arbor, MI; 1:1,000 dilution) at room temperature for 1 hr. Sections were then washed in phosphate-buffered saline (PBS) at pH 7.0 and incubated in PolyDetector HRP Label Reagent (Bio SB, Santa Barbara, CA) for 30 min. After further washing in PBS the sections were finally developed with DAB substrate-chromogen solution (Bio SB) and counterstained with Mayer's hematoxylin. Slides were then dehydrated through increasing concentrations of alcohol to xylene and coverslip mounted with Entellan (Merck, Darmstadt, Germany).

Urine and Bladder Tissue Cytokine Expression

Urine samples were collected in iced bowls for 4 hr. The collected urine was centrifuged at 3,000 rpm for 10 min at 4°C. The supernatants were promptly frozen at −80°C and kept until analysis. Frozen urine samples were thawed at room temperature for measurement of urine prostaglandin E2 by EIA Kit (Cayman Chemical). The cytokine concentrations for each time point were normalized to the creatinine concentration in urine and are expressed as the amount of cytokine in picograms excreted per milligram of creatinine.

Bladder tissue samples were excised and snap frozen in liquid nitrogen prior to storage at −80°C. Tissue samples were homogenized in ice-cold homogenization buffer consisting of 0.1 M phosphate, pH 7.4, containing 1 mM EDTA and 10 μM indomethacin. The homogenates were centrifuged at 14,000*g* for 30 min. The resulting supernatants were used for measurement of tissue PGE2 and IL-2. Measurements of tissue PGE2 and IL-2 by Prostaglandin E2 EIA Kit (Cayman Chemical), and Quantikine Rat IL-2 (R&D System, Minneapolis, MN), respectively. PGE2 and IL-2 concentrations in the tissue were normalized to the respective bladder weight of each rat.

Measurement of Systemic Tacrolimus Levels

Whole blood from three rats instilled with LFK was isolated and maintained at 4°C until analysis. The Microparticle Enzyme Immunoassay (MEIA II) for tacrolimus was performed on an IMx analyzer according to the package insert instructions supplied by the manufacturer (Abbott Laboratories, Abbott Park, IL). The assay has an analytical detection limit of 1.5 ng/ml.[14]

Statistical Analysis

Quantitative data are expressed as means ± standard error of mean. Statistical analyses were performed using *t*-test or one-way ANOVA, with Bonferroni post-test where applicable, with $P < 0.05$ considered significant.

RESULTS

CMG Responses to CYP, LP, or LFK Treatment

As shown in Table I and Figure 1A–D, on day 3, CYP treatment induced a reduction in ICI (61.4% and 58.1%, for control group and LP-treated group), which is one of the parameters accounting for bladder overactivity. CYP-induced overactivity was only suppressed in rats with LFK treatment (Table I and Fig. 1E,F; ICI 19.6% decrease). These results indicate the therapeutic effect of LFK on bladder overactivity.

Histological Responses to LPs and LFK Treatment

As shown in Table II, Figure 2B, CYP injection induced severe inflammatory reaction, edematous changes in the bladder mucosa and submucosal layers associated with a larger amount of inflammatory cells accumulation, as determined by the histopathological evaluation of tissue sections stained with hematoxylin and eosin. These inflammatory responses after CYP injection were not suppressed in groups without tacrolimus treatment (Fig. 2C). However, the CYP-induced inflammatory reaction was significantly decreased in the LFK-treated group (Fig. 2D) versus control groups (edema score and inflammatory cell score, 39.3% and 35.7% decrease, respectively). These results indicate that LFK treatment inhibits CYP-induced bladder inflammation.

EP4 Immunostaining After LPs and LFK Treatment

As shown in Figure 3, CYP treatment induced an increase in EP4 expression in the nucleus of bladder mucosa and inflammatory cells of submucosa regions (Fig. 3B), which were not suppressed in groups without tacrolimus treatment (Fig. 3C). However, the CYP effects were decreased in the LFK-treated group (Fig. 3D). These results indicate that LFK treatment inhibits CYP-induced EP4 upregulation in the bladder.

TABLE I. Effects of Control, LP, and LFK on CMG Parameters

	Day 1	Day 3	*P*-value (day 1 vs. day 3, *t*-test)
Intercontraction interval (min)			
Control	13.57 ± 0.90	5.24 ± 1.02	0.0013
LP	11.74 ± 1.18	4.92 ± 1.42	0.0100
LFK	13.13 ± 0.95	10.55 ± 1.95	0.1238
Contraction amplitude (cmH_2O)			
Control	46.14 ± 8.31	55.31 ± 12.26	0.5022
LP	36.79 ± 3.27	52.9 ± 9.9	0.1166
LFK	47.99 ± 5.25	36.85 ± 5.06	0.1085
Baseline pressure (cmH_2O)			
Control	3.78 ± 0.60	3.78 ± 1.22	0.9990
LP	2.30 ± 0.52	2.17 ± 0.53	0.8243
LFK	5.6 ± 0.4	3.93 ± 0.76	0.1224
Pressure threshold (H_2O)			
Control	8.14 ± 1.49	9.59 ± 2.34	0.5764
LP	6.26 ± 0.69	5.71 ± 1.11	0.7208
LFK	7.8 ± 0.8	6.14 ± 0.62	0.1390

Urine PGE2 and Bladder Tissue PGE2 and IL-2 Level on LP and LFK Treatment

As shown in Tables II and III, CYP treatment induced increases in the level of urine PGE2 and bladder tissue PGE2 and IL-2 levels, which were not suppressed in groups without tacrolimus treatment. However, the CYP effects were decreased in the LFK-treated group. These results indicate that LFK treatment inhibits CYP-induced increases in urine PGE2 and bladder tissue PGE2 and IL-2 levels.

Measurement of Systemic Tacrolimus Levels

Measurement of tacrolimus levels in the blood of rats instilled with LFK was performed. Blood analysis of three rats at the same time point as the day 3 cystometric analysis revealed that systemic levels of tacrolimus were below the detection limit of the (<1.5 ng/ml) most widely used biochemical assay for tacrolimus.

TABLE II. On Day 3, Effects of Sham, Control, LP, and LFK on Inflammation (Inflammatory Scoring and Edema Scoring), and Bladder IL-2 and PGE2 Levels

	Edema (N = 6)	Inflammatory cell	IL-2 level (N = 7, pg/μg protein)	PGE2 level (N = 7, pg/μg protein)
A: sham	0.5 ± 0.2	0.3 ± 0.2	0.003782 ± 0.00053	0.01126 ± 0.00327
B: control	2.8 ± 0.2	2.8 ± 0.2	0.013330 ± 0.01333	0.03955 ± 0.00985
C: LP	2.7 ± 0.2	2.7 ± 0.2	0.011390 ± 0.00228	0.03721 ± 0.00787
D: LFK	1.7 ± 0.2	1.8 ± 0.2	0.003532 ± 0.00150	0.00993 ± 0.00251

	Edema	Inflammatory cell	IL-2 level	PGE2 level
ANOVA, Bonferroni comparison test				
A vs. B	<0.001	<0.001	<0.01	<0.05
A vs. C	<0.001	<0.001	<0.05	>0.05
A vs. D	<0.01	<0.001	>0.05	>0.05
B vs. C	>0.05	>0.05	>0.05	>0.05
B vs. D	<0.01	<0.01	<0.01	<0.05
C vs. D	<0.05	<0.05	<0.05	<0.05

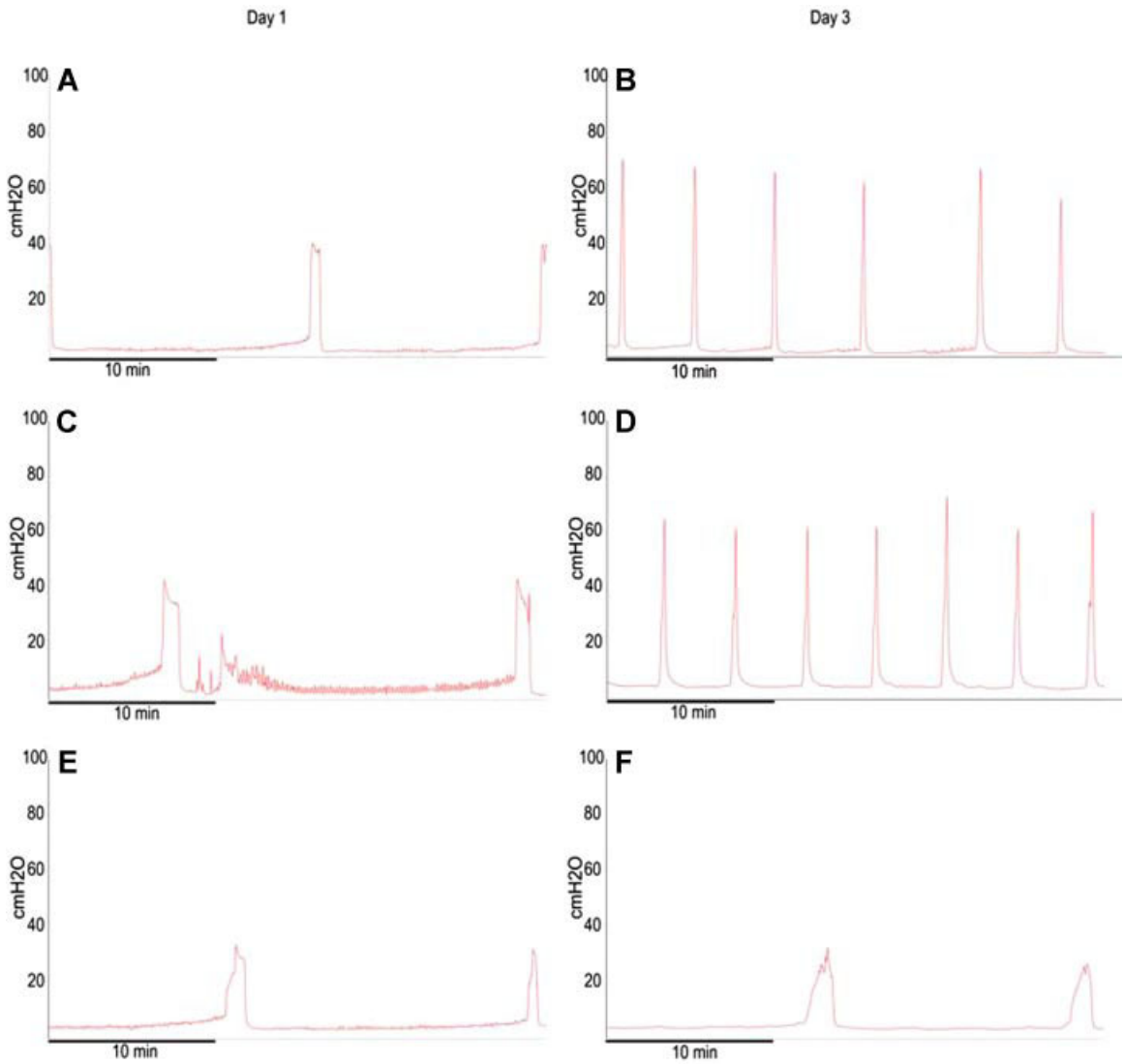

Fig. 1. Representative traces of in vivo continuous cystometrograms (CMG) in urethane anesthetized rats. CMG was performed in control (**A**,**B**), LP-treated rat (**C**,**D**), and LFK-treated rats (**E**,**F**). Both control and LP-treated rats showed a significant reduction in ICI on day 3 compared with day 1 CMG; however, the reduction in ICI was significantly reduced in the LFK-treated rat.

DISCUSSION

Tacrolimus is an effective immunosuppressive agent for organ transplantation as well as inflammatory disorders.[7,8] However, its systemic administration is associated with the risk of drug-related side effects such as hypertension, renal failure, and neurotoxicity. It is worthwhile to note that calcineurin inhibitors such as tacrolimus can provide targeted anti-inflammatory effect on topical use without the systemic side effects.[8,15] However, poor aqueous solubility of tacrolimus will present challenges in its formulation as its injectable formulation requires the use of surfactants.[9] Apart from solubility,

TABLE III. Effects of Sham, Control, LP, and LFK on Urine PGE2 Level

N = 7	Sham day 1	Control	LP	LFK	Sham day 3	Control	LP	LFK
PGE2/creatinine (pg/mg)	10.4 ± 2.4	11.8 ± 3.0	19.5 ± 6.1	23.2 ± 6.8	8.5 ± 1.2	148.1 ± 49.8	198.0 ± 55.0	68.7 ± 21.5
	Sham		Control		LP		LFK	
P-value (day 1 vs. day 3), *t*-test PGE2	0.5633		0.0312		0.0153		0.0218	

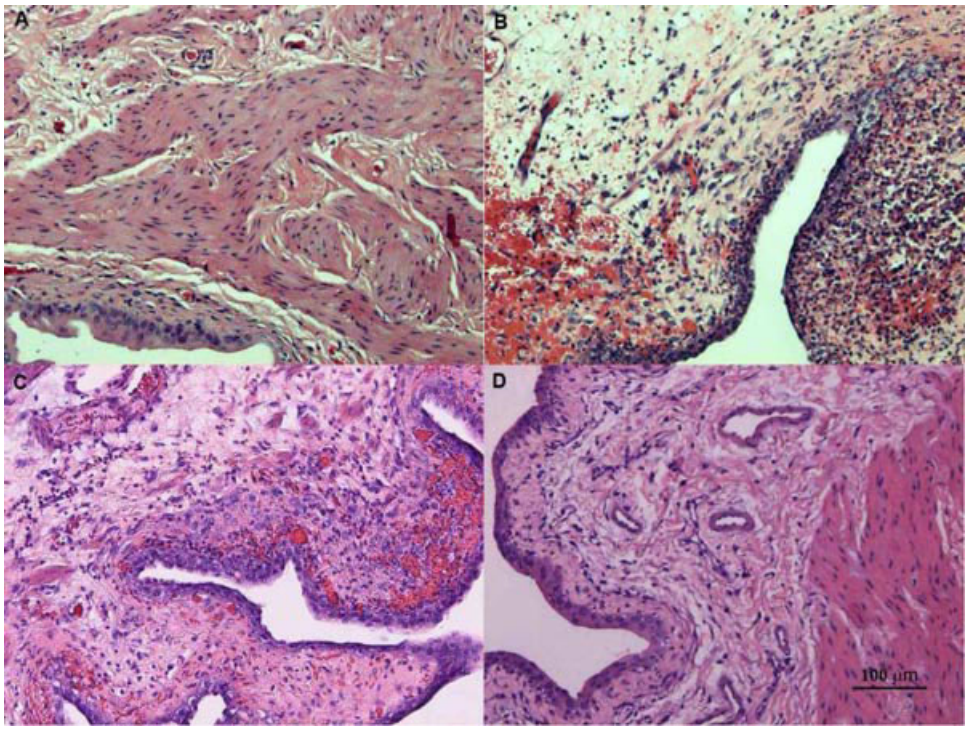

Fig. 2. Photomicrographs of bladder sections. CYP significantly induced inflammatory cells accumulation, edematous changes, and hemorrhage of the urothelium in control (**B**) and LP-treated rats (**C**) compared with the sham rat (**A**); however, the CYP effects were reduced in the LFK-treated rat (**D**). Magnification 100×.

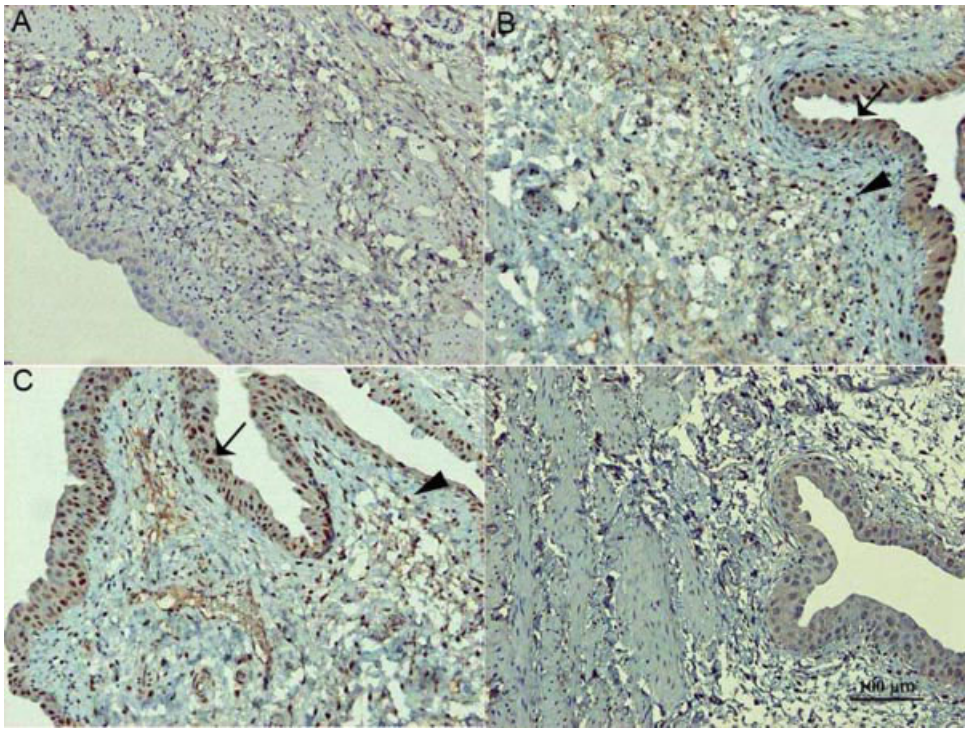

Fig. 3. EP4 staining in representative sections from the experimental rat bladder. CYP significantly induced increased EP4 staining in control (**B**) and LP-treated rats (**C**) compared with the sham rat (**A**); however, the CYP effects were reduced in the LFK-treated rat (**D**). Arrow, EP4-positive staining at the mucosal layer; arrowhead, EP4-positive staining at the submucosal layer; magnification 100×.

bladder uptake of tacrolimus will be hindered by its molecular weight (m.w. 822) as simple passive diffusion for bladder uptake is only seen with drugs with molecular weight of 400 such as thiotepa.[16] These physiochemical features of tacrolimus further accentuate the need of an efficient and safe delivery system for intravesical administration.[16]

Intravesical drug delivery using the versatile platform of LP nanoparticles offers several advantages, including universal carrier suitability for most lipophilic drugs, compatibility with urine, stable sustained release, and intracellular delivery.[17] The empiric success of this approach was reported from our group with liposomal formulation of botulinum toxin for uniform local deposition botulinum toxin in bladder without retention.[18] In the current study, retention of tacrolimus efficacy after encapsulation in LP was demonstrated through suppression of CYP-induced SHC by intravesical LFK treatment and attenuated bladder overactivity and inflammatory reaction.

Although the true mechanism of LFK on the bladder epithelium is not clear, LP encapsulating tacrolimus may be endocytosed into the bladder and tacrolimus may reach out from the adsorbed LP onto the urothelium. The results of our study thus suggest that LPs can significantly increase the solubility of tacrolimus and facilitate the absorption of high molecular weight drugs presumably by increased vesicular traffic promoted by the vesicle nature of LP itself.

There are several potential ways, by which tacrolimus could act following successful bladder uptake from LP, including either inhibition of its target molecule calcineurin with blockade of subsequent nuclear factor of activated T-cell (NFAT)-dependent gene transcription of cytokines in T cells and mast cells or interference with NFκB pathways.[19,20] Recent studies also suggest inhibition of peptide neurotransmitters from peripheral terminals underlying its efficacy following topical administration.[20] However, its main mechanism of action seems to remain inhibition of IL-2-dependent T-cell activation.[21] In our study, the elevation of bladder IL-2 concurred with the occurrence of bladder overactivity in SHC. IL-2 plays a role of an auto- and paracrine growth factor during the first 48–72 hr of T-cell activation.[22] Furthermore, tacrolimus is known to downregulate the cyclooxygenase-2 enzyme[23] and this effect was reflected in the significantly reduced immunostaining of EP4 receptors. In addition, urine PGE2 was also significantly decreased in the LFK-treated group compared to the control indicated that LFK modulated the CYP-induced inflammation by reducing the expression of PGE2 and EP4.

In this study, instillation of lipo-tacrolimus allowed maximum exposure of tacrolimus to the urothelium at the site of the initial immunologic engagement following acrolein-mediated bladder injury. The systemic tacrolimus levels measured at the time point of cystometry after instillation were below the detection limit of the most widely used clinical assay (<1.5 ng/ml). These results are consistent with earlier dermatologic studies, which reported nine times higher skin concentration of tacrolimus following topical application of liposomal tacrolimus lotion than that achieved with systemic administration.[15]

We have previously reported the therapeutic effects from intravesical instillation of empty LPs in an animal model of wounded bladder urothelium as well as patients with interstitial cystitis/painful bladder syndrome (IC/PBS).[24,25] However, in the current study, the treatment with single dose of intravesical LP could not resist the severe bladder inflammation and bladder overactivity caused by sustained release of acrolein in bladder following CYP injection. To circumvent this problem of the animal model, single bladder instillation of acrolein has been used in the past instead of intraperitoneal injection of CYP to evaluate the drugs currently approved by FDA for IC/PBS.[26]

In conclusion, bladder uptake of tacrolimus can achieve intravesical immunosuppression that can prove beneficial in bladder diseases involving aberrant immune component and/or inflammation such as after CYP. LPs are a promising vehicle for tacrolimus delivery and the encapsulated drug is able to interfere with early T-cell activation for inhibition of CYP-induced bladder inflammation and bladder overactivity as evident from changes in IL-2, PGE2, and EP4 levels in treated animals. These findings suggest that intravesical liposomal formulation and delivery of tacrolimus may provide a novel and promising method for the treatment of refractory inflammatory bladder conditions such as SHC and suppression of bladder overactivity.

REFERENCES

1. Grinberg-Funes DJ, Sheldon C, Weiss M. The use of prostaglandin F2 alpha for the prophylaxis of cyclophosphamide induced cystitis in rats. J Urol 1990;144:1500–4.
2. Klippel JH. Cyclophosphamide: Ovarian and other toxicities. Lupus 1995;4:1–2.
3. Cox PJ. Cyclophosphamide cystitis—Identification of acrolein as the causative agent. Biochem Pharmacol 1979;28:2045–9.
4. Sakthivel SK, Singh UP, Singh S, et al. CXCL10 blockade protects mice from cyclophosphamide-induced cystitis. J Immune Based Ther Vaccines 2008;6:6.
5. Tyagi P, Tyagi V, Yoshimura N, et al. Gender-based reciprocal expression of transforming growth factor-beta1 and the inducible nitric oxide synthase in a rat model of cyclophosphamide-induced cystitis. J Inflamm (Lond) 2009;6:23.
6. Smaldone MC, Vodovotz Y, Tyagi V, et al. Multiplex analysis of urinary cytokine levels in rat model of cyclophosphamide-induced cystitis. Urology 2009;73:421–6.
7. Migita K, Eguchi K. FK 506-mediated T-cell apoptosis induction. Transplant Proc 2001;33:2292–3.
8. Ebert AK, Rosch WH, Vogt T. Safety and tolerability of adjuvant topical tacrolimus treatment in boys with lichen sclerosus: A prospective phase 2 study. Eur Urol 2008;54:932–7.
9. Tamura S, Ohike A, Ibuki R, et al. Tacrolimus is a class II low-solubility high-permeability drug: The effect of P-glycoprotein efflux on regional permeability of tacrolimus in rats. J Pharm Sci 2002;91:719–29.
10. Giannantoni A, Di Stasi SM, Chancellor MB, et al. New frontiers in intravesical therapies and drug delivery. Eur Urol 2006;50:1183–93.
11. Chuang YC, Yoshimura N, Huang CC, et al. Intravesical botulinum toxin A administration inhibits COX-2 and EP4 expression and suppresses bladder hyperactivity in cyclophosphamide induced cystitis in rats. Eur Urol 2009;56:159–67.
12. Hu VY, Malley S, Dattilio A, et al. COX-2 and prostanoid expression in micturition pathways after cyclophosphamide-induced cystitis in the rat. Am J Physiol Regul Integr Comp Physiol 2003;284:R574–85.
13. Chuang YC, Yoshimura N, Huang CC, et al. Expression of E-series prostaglandin (EP) receptors and urodynamic effects of an EP4 receptor antagonist on cyclophosphamide induced overactive bladder in rats. BJU Int 2010 (in press). doi:10.1111/j.1464-410X.2010.09260.x
14. Ghoshal AK, Soldin SJ. IMx tacrolimus II assay: Is it reliable at low blood concentrations? A comparison with tandem MS/MS. Clin Biochem 2002;35:389–92.
15. Erdogan M, Wright JR, McAlister VC. Liposomal tacrolimus lotion as a novel topical agent for treatment of immune-mediated skin disorders: Experimental studies in a murine model. Br J Dermatol 2002;146:964–7.
16. Tyagi P, Chancellor M. Drug delivery and intravesical instillation. In: Kreder K, Dmochowski R, editors. The Overactive Bladder: Evaluation and Management, London: Informa Healthcare; 2007. pp 115–25.
17. Tyagi P, Tyagi S, Kaufman J, et al. Local drug delivery to bladder using technology innovations. Urol Clin North Am 2006;33:519–30, x.
18. Chuang YC, Pradeep T, Huang CC, et al. Urodynamic and immunohistochemical evaluation of intravesical botulinum toxin A delivery using liposomes. J Urol 2009;182:786–92.
19. Hamada N, Miyata M, Eto H, et al. Tacrolimus-eluting stent inhibits neointimal hyperplasia via calcineurin/NFAT signaling in porcine coronary artery model. Atherosclerosis 2010;208:97–103 [Epub July 28, 2009].
20. Inagaki N, Shiraishi N, Igeta K, et al. Depletion of substance P, a mechanism for inhibition of mouse scratching behavior by tacrolimus. Eur J Pharmacol 2010;25:283–9.

21. Trompeter R, Filler G, Webb NJA, et al. Randomized trial of tacrolimus versus cyclosporin microemulsion in renal transplantation. Pediatr Nephrol 2002;17:141–9.
22. Crispín JC, Tsokos GC. Transcriptional regulation of IL-2 in health and autoimmunity. Autoimmun Rev 2009;8:190–5.
23. Jespersen B, Thiesson HC, Henriksen C, et al. Differential effects of immunosuppressive drugs on COX-2 activity in vitro and in kidney transplant patients in vivo. Nephrol Dial Transplant 2009;24:1644–55.
24. Fraser MO, Chuang YC, Tyagi P, et al. Intravesical liposomes administration: A novel treatment for hyperactive bladder in the rat. Urology 2003;61:656–63.
25. Chuang YC, Lee WC, Lee WC, et al. Safety and efficacy of intravesical liposome versus oral pentosan polysulfate in patients with interstitial cystitis/painful bladder syndrome. J Urol 2009;182:786–92.
26. Kalota SJ, Stein PC, Parsons CL. Prevention of acrolein-induced bladder injury by pentosanpolysulfate. J Urol 1992;148:163–6.