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Effects of menstrual cycle on gene transfection through mouse vagina for DNA vaccine

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ABSTRACT

Human immunodeficiency virus (HIV) infections mainly occur through the vaginal and rectal mucosal membranes. In the present study, to develop a DNA vaginal vaccine against viral and bacterial infections, the effects of the menstrual cycle on DNA transfection through the vaginal mucosa in female mice and transfection enhancement by electroporation, a chelating agent, cell-penetrating peptides (CPP) and nuclear localizing signals (NLS) were investigated. The transfection efficiencies of a marker plasmid DNA (pDNA), pCMV-Luc, on the vaginal mucosal membrane in mice at the stages of metestrus and diestrus were significantly higher than those at the stages of proestrus and estrus. The gene expression was markedly enhanced by electroporation and by pretreatment with the chelating agent. The highest level of expression was obtained by 2 h pretreatment with 5% citric acid solution combined with electroporation with 15 pulses at 250 V/cm for 5 milliseconds (ms). Furthermore, a synergistic promoting effect on pDNA transfection was obtained by co-administration of CPP, the Tat peptide analog, and NLS, the NF-κB analog. These results indicate that effective DNA vaccination administered through the vaginal tract is possible by selecting the menstrual stage and overcoming the mucosal barrier using a combination of methods that promotes uptake.

1. Introduction

There is an urgent need for the development of safe, effective, stable and inexpensive vaccines against severe viral infections, including HIV and SARS. The majority of HIV infections currently occur through heterosexual intercourse and transmission through the vaginal mucosa (De Schryver and Meheus, 1990; Quinn, 1996). The accumulated evidence suggests an important role for systemic cell-mediated immune responses against HIV-1 gag in the prevention of infection and protection against AIDS (Koup et al., 1994; Borrow et al., 1994, 1997). However, the presence of SIV/HIV-specific cytolytic T-lymphocytes (CTL) throughout the vaginal and uterine mucosal membranes, which are the sites of viral entry, as well as in the draining lymph node (LN) is well-documented and this may be associated with protection (White et al., 1997; McChesney et al., 1998; Kaul et al., 2000). Therefore, a successful HIV vaccine will most likely need to elicit long-term cell-mediated immunity at the site of viral entry, the vaginal mucosa, and the draining LN, as well as systemically (Singh et al., 2001). Many studies have investigated induction of active immune responses at various mucosal surfaces, particularly the gastrointestinal tract, but relatively few studies have examined antibody titer in the genital secretions after antigen delivery (Marx et al., 1993; Parr et al., 1988). Thus, the most effective methods for stimulating immunity at the vaginal surface have yet to be identified. However, from the studies to date it appears that mucosal delivery of antigen produces a better local response than systemic delivery (Marx et al., 1993; Parr et al., 1988). Controlling the duration of local exposure may also enhance local immunity (Wassen et al., 1996; Tammy et al., 1998), although this hypothesis has not been fully investigated. Although traditional vaccines have been comprised of several proteins, such as live attenuated viruses, killed bacteria and components of viral proteins, much attention has recently been focused on DNA vaccination (Otten et al., 2005; Vajdy et al., 2004; Mollenkopf et al., 2004). DNA-based vaccines provide a promising new approach for AIDS immunotherapy, as they offer several advantages over conventional immunization using proteins. The most important benefit is the induction of strong local mucosal immunity and CTL immune responses in addition to systemic immunity. The ruggedness and relative simplicity of DNA offers the potential for improved vaccine stability and reduced vaccine production costs. Moreover, compared to attenuated viruses as delivery vehicles for HIV genes,

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plasmid DNA offers a safe alternative (Wang et al., 1998; MacGregor et al., 1998).

To achieve significant levels of immunity in humans and large animals, DNA delivery methods often require very high doses of plasmids and multiple boosts (Van Drunen Littel-van den Hurk et al., 2000; Le et al., 2000; MacGregor et al., 1998; Babiuk et al., 2003). Thus the potentiation of efficiency of DNA vaccines in humans is required. Even at high doses, DNA vaccines in humans have failed to be efficacious. Therefore, more potent forms of the DNA vaccines themselves and/or more effective means of delivery and potent adjuvants must be developed for the technology to realize its potential.

One reason for the low effectiveness of DNA vaccines in humans could be insufficient membrane permeability and cellular uptake of pDNA, resulting in poor gene expression. *In vivo* electroporation may allow increased gene expression by enhancing cellular uptake of plasmids by the application of short electrical pulses that transiently permeabilize the cellular membranes. Electroporation may also enhance nuclear uptake. It has been clearly shown that DNA delivery into the muscle tissue of mice or large animals followed by electroporation, strongly increases gene expression and immune responses elicited by the DNA vaccines (Zucchelli et al., 2000; Heller et al., 2001; Dujardin et al., 2001; Babuik et al., 2003). Furthermore, when used for DNA delivery in the skin, electroporation amplifies gene expression with both intradermal and topical administration of pDNA.

Recently, a cellular internalization method using short peptides derived from protein-transduction domains has attracted much attention. Several cell-penetrating peptides (CPPs), such as HIV-1 Tat fragments, less than 30 amino acid residues in length, are capable of crossing a plasma membrane (Futaki et al., 2001; Morris et al., 2001; Schewarze et al., 1999). In addition, they can deliver their associated molecules into cells. The Tat peptide has been reported to be capable of delivering β-galactosidase (120 kDa) to various organs when administered intraperitoneally to mice.

Gene transfer systems based on lipoplexes or polyplexes have gained wide acceptance over the last decade as gene transfer vectors. However, at present their usefulness and applications as therapeutic devices are limited by the transient, low levels of gene expression observed in vivo. One of the limiting steps responsible for a low gene expression with these non-viral vectors resides in inefficient intracellular trafficking of DNA from the cytoplasm to the nucleus. To overcome cytoplasmic degradation of the gene via effective transport into the nucleus and to improve the efficiency of gene expression, the use of nuclear localization signal (NLS) peptides for non-viral gene transfer has been widely investigated (Zabner et al., 1995; Ohno et al., 1998). Except during mitosis, macromolecules such as proteins or nucleic acids cannot enter the nucleus through the nuclear pores. The intranuclear transfer of cellular and viral proteins, DNA, and/or RNA occurs by means of an energy-dependent mechanism which involves peptidic NLS sequences that bind to structures called the nuclear pore complex (NPC) via transport receptors such as importins α/β (Boulanger et al., 2005). To date, the literature indicates that the NLS approach has potential for improving DNA nuclear delivery and expression with non-viral vectors.

The vaginal absorption of relatively large and water soluble compounds, such as peptides and proteins, has been systemically determined in rats to be very poor and significantly influenced by the menstrual cycle (Okada et al., 1983a, 1984; Okada, 1991). Studies have shown that after vaginal administration of an insulin suppository in rats, a slight decrease in the glucose level is observed during proestrus, whereas a marked decrease is observed during metestrus and diestrus. Furthermore, the absorption of water soluble peptides, such as insulin and leuproreline acetate, through the vaginal membrane is much higher during metestrus and diestrus than that during proestrus and estrus (Okada et al., 1983b). However, the vaginal absorption of nucleotides, such as RNA and DNA, has not previously been reported.

In the present study, we investigated the effects of the menstrual cycle, electroporation, citric acid as a chemical absorption enhancer and co-administration with a CPP and NLS on the level of gene expression of marker plasmid DNAs administered through the vaginal membrane in mice.

2. Materials and methods

2.1. Materials

The electroporator (Electro square porator T820: BTX Genetronics, San Diego, CA, USA) was kindly supplied by Hisamitsu Pharmaceutical Co., Inc. The Luciferase Assay System (Promega Co., Ltd., USA) was used for determining luciferase activity. All other chemicals were of the finest grade available.

2.2. Plasmid preparations

Plasmid DNA (pCMV-Luc, 7607bp), which consisted of a luciferase cDNA fragment subcloned into pcDNA3.1 at the Hind III and BamH1 sites, was amplified in *E. coli* (DH5α) and purified using an Endfree Plasmid Maxi kit (QIAGEN, USA) followed by ethanol precipitation and dilution in Tris/EDTA buffer. The DNA concentration was measured by UV absorption at 260 nm. In addition, pEGFP-N1 plasmid (4.7 kb, BD Biosciences Clontech, USA), which codes for green fluorescence protein, was used for fluorescence microscopy (Axiovert 200 M, Carl Zeiss, USA).

2.3. Peptide carriers

The Tat analog, which consists of Cys-Gly-NH_2 added to the N terminus of HIV-Tat (48–57), and the NF-κB analog, which consists of Gly and Cys-Gly-NH_2 added to the C and N termini of NF-κB p50, were synthesized as CPP or NLS peptide gene vectors using the Fmoc-solid-phase peptide synthesis method with a ABI 433A peptide synthesizer (Applied Biosystems, Japan) (Table 1). Both analogs were then purified by reverse-phase HPLC. The molecular weight of each analog was determined by matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry (MALDI-TOFMS): Tat analog, 1396.1; NF-κB analog, 1130.36. Peptide carrier/pDNA (weight ratio: 10/1) complexes were prepared by mixing pDNA with each peptide and incubating at 4 °C for 30 min.

2.4. Animals

Female 6- to 8-week-old ICR mice were obtained from SLC (Shizuoka, Japan). The mice were housed under standard conditions of temperature (22–24 °C), humidity (40–60%) and 12-h light/12-h dark-cycles with the light period stating at 08:00 h. Food and water were supplied *ad libitum*. The animal experiments were carried out in accordance with a protocol approved by the Animal Care and Ethics Committee of Tokyo University of Pharmacy and Life Sciences (TULPS). The estrous stage of mice was determined through daily morning microscopic observation of vaginal smears taken with a

Table 1
Structure of synthetic Tat and NF-κB analogs

Peptides	Sequence
Tat analog	Gly-Arg-Lys-Lys-Arg-Arg-Gin-Arg-Arg-Arg-Cys-Gly-NH_2
NF-κB analog	Gly-Gin-Arg-Lys-Arg-Glu-Lys-Cys-Gly-NH_2

swab and stained with Giemsa solution. Stained cells were carefully examined and the estrous stage of each mouse identified as the diestrus, proestrus, estrus, or metestrus stage according to a previously established protocol (Okada et al., 1983a).

2.5. Electroporation

Mice in the estrous stage underwent insertion into the vaginal tract of a cotton ball soaked in 2–10% citric acid aqueous solution (about 100 μL). The cotton ball was removed 2 h later and the naked pDNA (20 μg) or peptide carrier (200 μg)/pDNA (20 μg) complexes then administered into the vaginal tract using a micropipet. The vaginal membrane was electroporated at the site of pDNA administration, using an electroporator (Electro square porator T820: BTX Genetronics, San Diego, CA, USA). A custom-designed needle electrode, consisting of two parallel needles 5 mm in length and 5 mm apart, was used to apply 5–30 pulses of electricity at 250 V/cm for 5–20 ms.

2.6. Luciferase assay

At 3–48 h after administration of the pDNA, the mice were sacrificed and then the vaginal membranes resected, homogenized in lysis buffer (0.05% Triton X, 2 mM EDTA, 0.1 M Tris) at a volume-to-weight ratio of 4 mL lysis buffer/g of each organ, and processed three times with freezing (−80 °C) and thawing. The homogenate was then centrifuged at 14,000 rpm for 40 min. The luciferase activity in 10 μL of the supernatant was measured using a commercial luciferase assay kit (Promega Co., Ltd., USA). Each measurement was carried out for 60 sec in a luminometer (MicroLumat Plus LB96V; Berthold, Germany). The protein concentration of each sample was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). The luciferase activity was normalized to each sample's protein concentration and expressed as RLU/mg protein.

2.7. Histological staining of vaginal tissue

To examine the vaginal mucous membranes of the mice at the various estrous stages, 10 μm frozen sections of vagina were fixed in cold acetone and washed with PBS solution three times and then stained in hematoxylin and eosin. Sections were observed under a light microscope.

2.8. Observation of site of gene expression

To examine the site of expression of the gene within the vagina, paraffin sections of vagina after administration using electroporation (15 pulses, 250 V/cm, 5 ms) of naked pEGFP (40 μg) or peptide carrier (200 μg)/pEGFP (20 μg) complexes were prepared. The slides were washed and mounted with Fluoroment-G and observed by fluorescence microscopy.

2.9. Statistical analysis

All values are expressed as mean ± S.E. Statistical analysis of luciferase activities was performed using an unpaired Student's *t*-test. Statistical significance was defined as $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results and discussion

3.1. Enhancement of gene expression with electroporation

The luciferase activity in the mouse vaginal mucosal membrane after a single vaginal administration of pCMV-Luc followed by electroporation with 15 electrical pulses of 250 V/cm for 5 ms increased gradually to a peak level at 18–24 h after administration (Fig. 1a). In this experiment, the vaginal membrane was pretreated with 5% citric acid aqueous solution for 2 h to overcome the mucosal barrier to hydrophilic compounds. Following this experiment, luciferase activity after transfection of pDNA was determined 24 h after vaginal administration of pDNA.

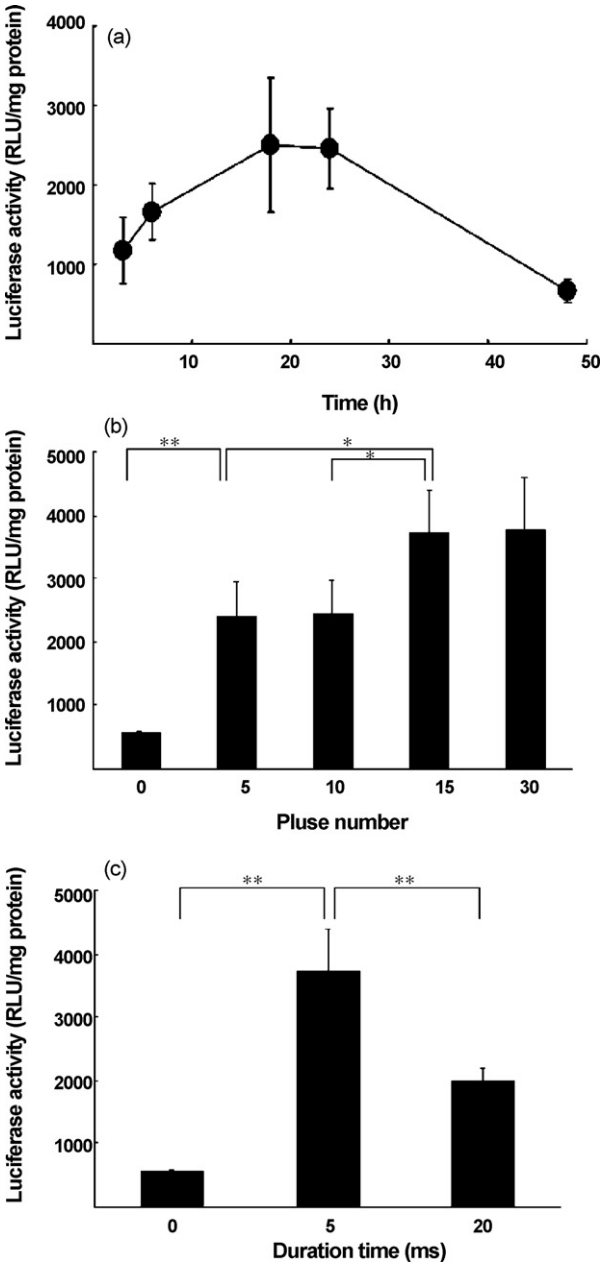

Fig. 1. Effect of electroporation on transfection of pCMV-Luc after vaginal administration in diestrous mice. Luciferase activity in the vaginal mucosal membrane was determined after vaginal administration of pCMV-Luc (20 μg) at diestrus via electroporation in mice that had undergone vaginal pretreatment with 5% citric acid solution for 2 h. (a) Time-course of luciferase activity following electroporation with 15 electrical pulses of 250 V/cm and 5 ms each, (b) influence of pulse number in the application of short electrical pulses on the vaginal membrane, (c) luciferase activity with various pulse durations. Each data point represents the mean ± S.E. ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, (*t*-test).

Fig. 1b shows the influence of pulse number in the application of short electrical pulses on the vaginal membrane. The expression of pCMV-Luc significantly increased with an increase in pulse number, and was maximal when 15 or more pulses were used. The vaginal

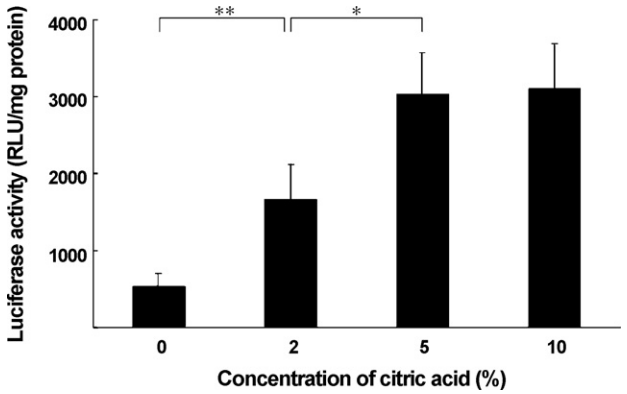

Fig. 2. Effects of pretreatment with citric acid solution on transfection of pCMV-Luc into vaginal mucosa in diestrous mice. Luciferase activity in the vaginal mucosal membrane was determined 24 h after vaginal administration of pCMV-Luc (20 μg) at diestrus via electroporation (250 V/cm, 5 ms, 15 pulses) in mice pretreated with 2, 5, or 10% citric acid solution for 2 h. Each data point represents the mean ± S.E. ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, (t-test).

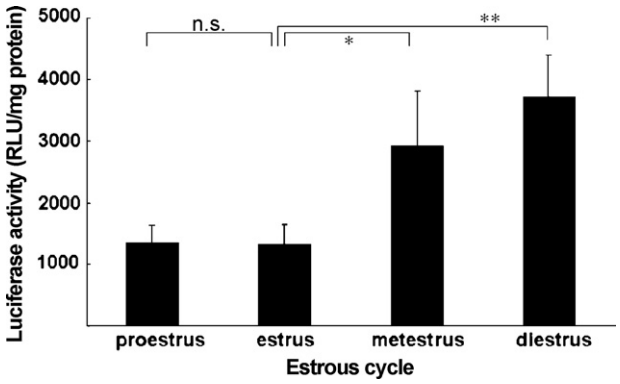

Fig. 3. Effects of estrus cycle on transfection of pCMV-Luc into vaginal mucosa in diestrous mice. Luciferase activity in the vaginal mucosal membrane was determined 24 h after vaginal administration of pCMV-Luc (20 μg) at various estros stages via electroporation (250 V/cm, 5 ms, 15 pulses) in mice pretreated with 5% citric acid solution for 2 h. Each data point represents the mean ± S.E. ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, n.s.: $p > 0.05$, (t-test).

membrane showed slight degeneration on the surface upon application of 30 pulses. Therefore, 15 pulses was selected as the optimal pulse number for electroporation in subsequent experiments.

The effect of the duration of electrical pulse was also assessed after administration of pDNA to the mouse vaginal membrane. Luciferase activity significantly increased by electroporation for 5 ms, however activity obviously decreased with 20 ms (Fig. 1c). Macroscopic damage was also subsequently observed when 20 ms was used.

In general, electroporation relies on the application of a relatively high-intensity electric field (typically > 100 V/cm) delivered in very short pulses (μ-ms duration). These conditions induce reversible destabilization and permeabilization of the cell plasma membrane enabling easier access of drugs and genetic material into cells (Bejjani et al., 2007). Damage during electroporation may occur by overheating, irreversible dielectric breakdown and associated necrosis by large electrical voltages and/or extended electric pulses. Therefore, the electroporation parameters (i.e., electrode design, field strength, and pulse duration) need to be specifically adapted to the tissue or cells in question in order to obtain optimal gene transfer. In a previous study, a gold-plated rod-like electrode was placed on either side of a proptosed eye, i.e., normal to the cornea, and used to deliver 8 pulses at 200 V/cm of 10 ms dura-

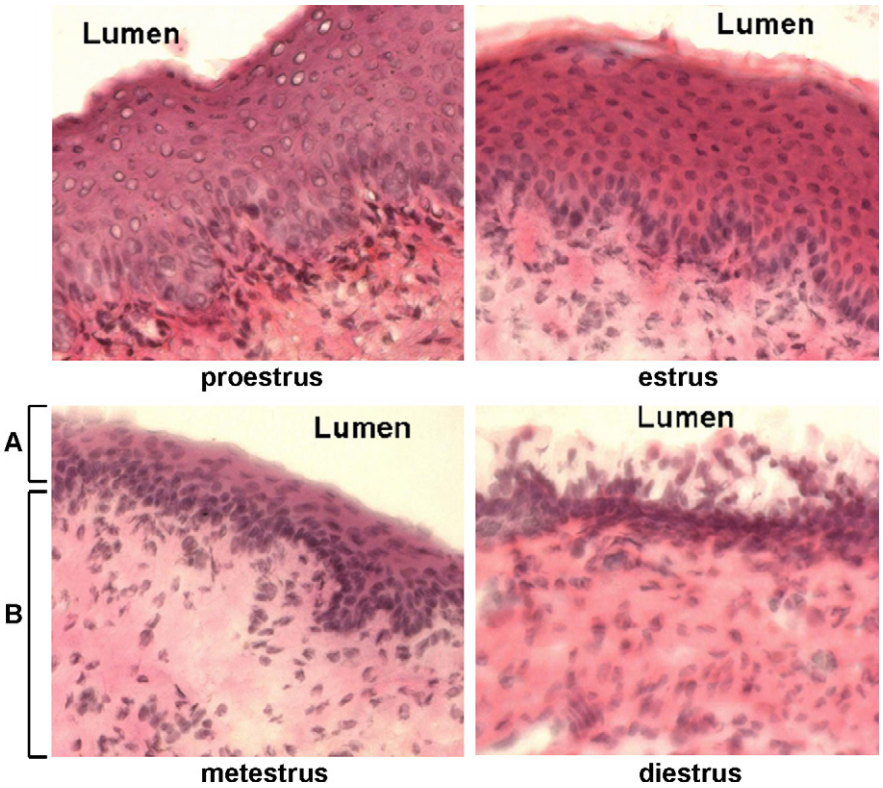

Fig. 4. Histological observation of section of the vaginal mucosal membrane in mice during different stages of the estrous cycle. The stage of the estrus cycle of the mice was determined using a morning smear test. Vaginal tissue was collected and 10 μm frozen sections stained with hematoxylin and eosin. A: epithelium, B: stroma (subepithelium), Lumen: vaginal lumen.

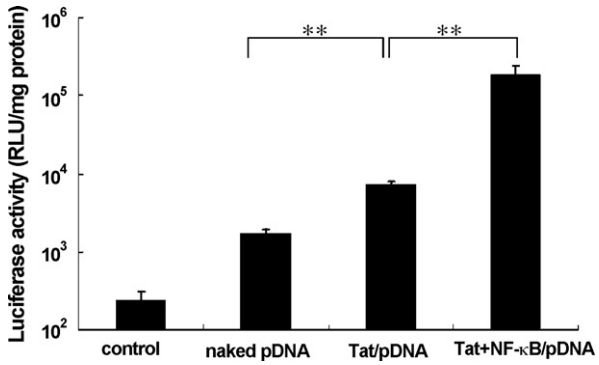

Fig. 5. Luciferase activity in the vaginal membrane after vaginal administration of pCMV-Luc with Tat and NF-κB analogs. Mice at diestrus received intravaginal administration of pCMV-Luc (20 μg) and complexes with Tat analog (200 μg) or NF-κB analog (200 μg), using electroporation (250 V/cm, 5 ms, 15 pulses). Mouse vaginas had been pretreated with 5% citric acid solution for 2 h followed by electroporation. Each data point represents the mean ± S.E. ($n = 4$). $^{**}p < 0.01$ (t-test).

tion (Blair-Parks et al., 2002). Using these parameters, no tissue damage, such as corneal edema or inflammation, was reported. Furthermore, the highest level of expression obtained using this methodology was 30-fold higher than those obtained using the lipofectin delivery system. In another transdermal study, histological analysis of skin damage due to gene transfer was performed on days 1 and 7 after *in vivo* electroporation, with the skin damage seen on day 1 disappearing by day 7 after electroporation (Maruyama et al., 2001). The results of the present study indicate that electroporation is extremely useful for gene delivery into the mouse vaginal mucosal membrane and that the optimal conditions for greatest efficacy with minimal local irritation are 15 pulses of 250 V/cm for 5 ms each. For human clinical studies these conditions would need to be re-optimized for different electrodes.

3.2. Effect of citric acid pretreatment on gene expression

Fig. 2 shows the efficiency of transfection into the mouse vaginal mucosal membrane after a single vaginal administration of pCMV-Luc followed by electroporation of 15 pulses of 250 V/cm and 5 ms each after a 2 h pretreatment with various concentrations of citric acid aqueous solution. As the concentration of citric acid aqueous solution increased, the level of luciferase activity also increased. No significant difference in transfection efficiency was noted between 5% and 10% citric acid (Fig. 2). Thus, pretreatment with 5% citric acid aqueous solution is optimal and very useful for gene delivery into vaginal mucosa. Our previous experiment on vaginal absorption of peptides found that citric acid induced transient and reversible opening of the tight junctions in the vaginal mucosal membrane and increased the vaginal absorption of several kind of peptides (Okada et al., 1983a). The present experiment using pDNA has also proved absorption enhancement through the intercellular route by citric acid. Citric acid pretreatment modulates the mucosal tight junctions between the mucosal epithelial cells and could overcome the mucosal barrier before DNA cellular transfection.

3.3. Effect of estrous cycle on gene expression

The estrous cycle of mice is completed in four to five days, although the timing of the cycle may be influenced by exteroceptive factors such as light, temperature, nutritional status and social relationships. The cycle is roughly divisible into four stages, proestrus, estrus, metestrus and diestrus. To examine the transfection efficiency into vaginal mucosa during the estrous cycle, the vaginal membrane was pretreated with 5% citric acid solution for 2 h, then

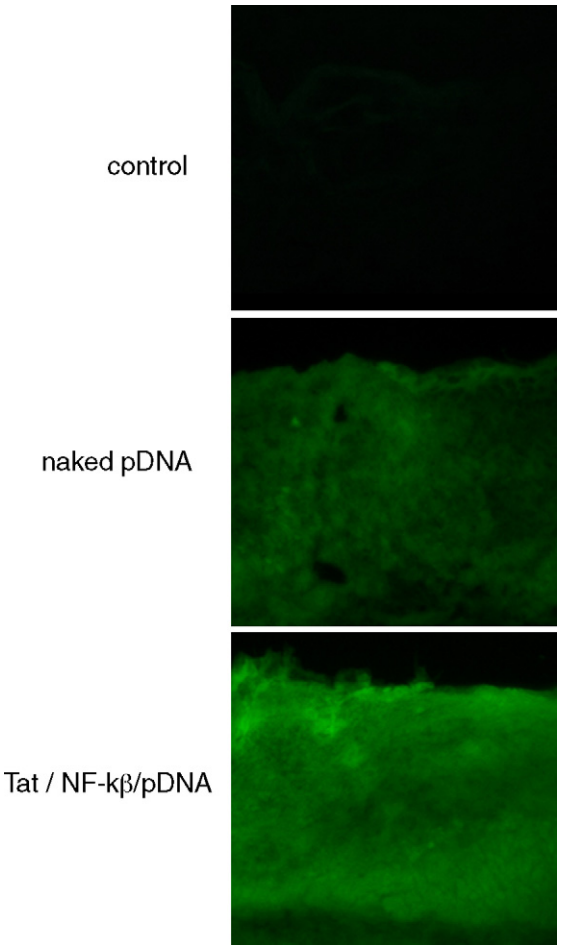

Fig. 6. GFP expression in vaginal mucosal membrane after administration of pEGFP. GFP expression in the vaginal mucosal membrane was observed 24 h after vaginal administration of pEGFP (20 μg) complexed with Tat analog (200 μg) or NF-κB analog (200 μg) at diestrus. Mouse vaginas had been pretreated with 5% citric acid solution for 2 h followed by electroporation (250 V/cm, 5 ms, 15 pulses). Vaginal tissue was collected and 10 μm frozen sections were examined by fluorescence microscopy.

electroporated with the naked pDNA into the vaginal membrane using optimized conditions at the four stages of proestrus, estrus, metestrus and diestrus. The estrous stage was assessed by daily morning microscopic observation of vaginal smears taken as a swab and stained with Giemsa solution and the pDNA was electroporated at around 09:00 in the morning following a smear check. Luciferase expression in the vaginal mucosa at each estrous stage was measured after 24 h. The transfection efficiency was clearly affected by the estrous cycle. As shown in Fig. 3, at the metestrus and diestrus, luciferase gene expression was 3-fold higher than at the proestrus and estrus. The mucosa of the vagina consists of epithelial cell layers that form a barrier to absorption of water soluble and large molecules. Histological observation (Fig. 4) indicated the difference in transfection efficiency at the four menstrual stages might be explained by a change in the membrane structure. At metestrus and diestrus, these epithelial cell layers are very thin compared with those at the other stages, and at diestrus they are extremely porous. It has been reported that the immune-associated cells in the vaginal submucosal membrane increase at diestrus (Zhao et al., 2003). Thus it was expected the DNA vaccination at diestrus, the late luteal phase and early follicle phase in humans, would be most suitable for practical therapy. Thereafter, subsequent experiments were performed during diestrus.

3.4. Enhancement of gene expression by the addition of Tat analog and NF-κB analog

To promote the efficiency of pDNA entry into the nuclei in the cells and consequently expression of the luciferase gene, the effects of the CPP and NLS analog peptides synthesized in our laboratory were evaluated. These peptides were complexed with pCMV-Luc and then administered into vaginal mucosa that had been pretreated with 5% citric acid solution for 2 h and electroporated during diestrus. Luciferase activity of the vaginal mucosa was then measured 24 h later. As shown in Fig. 5, the luciferase activity of mucosa treated with the Tat analog/pDNA complex was 3-fold higher than that treated with pDNA alone. Furthermore, a Tat analog/NF-κB analog/pDNA complex induced significantly greater luciferase gene expression in the vaginal mucosa than the Tat analog/pDNA complex. This level of expression was also statistically higher than those obtained by the widely used non-viral vectors, polyethyleneimine (PEI, Branched, Mw 76 kDa; Sigma–Aldrich Co., USA) and Lipofectamine® (Invitrogen Japan K.K., Japan) (data not shown). These results suggest that the Tat and NF-κB analogs facilitate the transfection of pDNA possibly by increasing both cell penetration and translocation into the nuclei. Furthermore, GFP expression produced by the Tat and NF-κB analogs/pEGFP complexes was also promoted to a greater extent than that by naked pDNA (Fig. 6). The exact mechanism of this potentiation of gene expression is unclear at the present time. However, these CPP and NLS analogs are promising as potentiators for DNA vaccine.

4. Conclusions

The transfection efficiency of plasmid DNA into vaginal mucosa via electroporation is strongly influenced by the estrous cycle, with higher luciferase gene expression observed during metestrus and diestrus. Electroporation itself enhanced luciferase gene expression though vaginal administration. Furthermore, citric acid, which is known to produce reversible dissociation of the tight junctions in mucosal membranes, also increased gene expression following electroporation of the marker gene though the vaginal membrane. The optimal conditions for gene transfection in the mice were a 2 h pretreatment with 5% citric acid solution followed by electroporation consisting of 15 pulses at 250 V/cm for 5 ms each though the vaginal mucosa at the stage of diestrus. The Tat analog and NF-κB analog synthesized by our laboratory statistically significantly promoted the transfection of pDNA, possibly though facilitating the cell penetration and nuclei localization. In the future, because electroporation raises concerns regarding safe use in humans, more non-invasive methods such as needle-free injection or microneedle injection rather than electroporation should be used. Our results provide useful insights into the vaginal mucosal delivery of pDNA.

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