Effect of Nefiracetam, a Neurotransmission Enhancer, on Primary Uroepithelial Cells of the Canine Urinary Bladder

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Repeated oral treatment of dogs with a high dose of nefiracetam is reported to induce hemorrhagic lesions in the urinary bladder. To delineate its pathogenesis, we established the primary culture of uroepithelial cells of the canine urinary bladder, and then explored the effect of nefiracetam on the cultured cells. Uroepithelial cells scraped from the connective tissues of the urinary bladder of naive dogs were suspended in the minimum essential medium containing dispase, and then resuspended in the keratinocyte medium to be 6.0–7.0 × 10⁵ cells/ml. Afterward, they were added to an apical chamber with a 12-mm transwell filter, cultured for three days, and recultured in the keratinocyte medium containing 1 mM CaCl₂ for 20 days. Microscopically, these cultured cells consisted of three cell layers with high transepithelial electric resistance (TER; > 10,000 ohm-cm²). Immunofluorescence observations revealed ZO-1 and E-cadherin bands, and electron microscopic examinations displayed the superficial cells with the assembly of tight junctions. When the effect of nefiracetam and its five main metabolites (M-3, M-10, M-11, M-18, and M-20) on TER and the ZO-1 band was assessed using cultured cells, only M-18 significantly reduced TER in the coculture for 48 h or more. Both M-10 and M-18 exhibited a deformation of uroepithelial cells and a slight reduction of the ZO-1 band from 120 h later. In conclusion, this culture system possesses both functional and morphological features of the uroepithelium reflected in vivo, and M-18 may play a pivotal role in the impairment of uroepithelial cells, leading to the onset of the urinary bladder lesion in dogs due to nefiracetam.

Key Words: nefiracetam; metabolites; canine urinary bladder; primary culture; uroepithelial cells; transepithelial electric resistance; morphology.

N-(2,6-Dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide (nefiracetam), a pyrrolidine derivative, has been shown to improve experimentally induced amnesia (Abe et al., 1994; Hiramatsu et al., 1992; Nabeshima et al., 1994; Sakurai et al., 1989). In humans, nefiracetam is under development for treatment of the sequelae of cerebrovascular disorders and senile dementia of the Alzheimer type, and no severe adverse effects have been reported so far (Murasaki et al., 1994; Otomo et al., 1994). In toxicological studies, however, repeated oral administration of a high dose of this compound induced urinary bladder injuries in dogs (Kashida et al., 1996), but not in rats (Jindo et al., 1994) or monkeys (unpublished data). Namely, when administered orally to dogs over one week, nefiracetam at 300 mg/kg/day caused epithelial ulceration with edema and hemorrhage in the lamina propria and muscle layer of the urinary bladder. In investigations of biotransformation of nefiracetam in several species including humans, it was found to be extensively metabolized, and more than 20 metabolites were observed in serum, urine, and the liver (Sudo et al., 1988). The major metabolic pathways of nefiracetam are shown in Figure 1. The urinary excretion of the main metabolites following oral treatment with nefiracetam at 30 mg/kg was: M-3 (35.9%, 0.86 mM), M-4 (6.7%, 0.16 mM), and M-11 (10.0%, 0.23 mM), and M-18 (21.2%, 0.37 mM) for rats, M-3 (35.9%, 0.86 mM), M-4 (4.0%, 0.09 mM) for dogs, and M-3 (21.2%, 0.37 mM), M-4 (8.7%, 0.20 mM), and M-11 (4.3%, 0.10 mM) for monkeys. The metabolic pattern in human urine is essentially similar to that in monkeys (unpublished data). These metabolic data raise the possibility that M-10 may be one of the key metabolites for dogs.

The uroepithelium comprises a multiple cell layer including the basal cell layer that attaches to connective tissues, the intermediate cell layer that is one to two layers in thickness, and the superficial cell layer, which contains highly differentiated cells (umbrella cells) that line the luminal surface of the bladder (Hicks, 1975). The umbrella cells play a crucial role in the bladder barrier function. High resistance tight junctions seen in the umbrella cell layer provide an effective barrier to the diffusion of solutes via the paracellular pathway and help maintain the biochemically and functionally distinct apical and basolateral surface domains of polarized epithelia (Gumbiner, 1987; Stevenson et al., 1988). In addition, the apical plasma membrane of the umbrella cell is highly impermeable to water (Chang et al., 1994; Negrete et al., 1996) and is covered by an adhering glycosaminoglycan (Parsons et al., 1991). The umbrella cells also possess a sodium channel within their apical plasma membranes along with other ion transport systems...
(Lewis and Diamond, 1976; Smith et al., 1998). These channels regulate the transcellular ionic flux across the epithelium and may be important in maintaining the large osmotic gradient between urine and vicinal tissues. As a result of these specializations, the umbrella cell forms one of the tightest and most impermeable barriers in the body.

More recently, a method to evaluate the barrier function using primary uroepithelial cells of the rabbit urinary bladder has been reported (Truschel et al., 1999). This system had physiological characteristics of intact urinary bladder epithelia including the presence of an apical umbrella cell layer, impermeability to water and urea, and development of high transepithelial electrical resistance (TER; >8000 ohm-cm²). To the best of our knowledge, there have been no reports dealing with the barrier function in the primary urinary bladder cells of dogs. Therefore, we developed primary cultured uroepithelial cells of the canine urinary bladder and used them to assess the effects of nefiracetam and its five main metabolites (M-3, M-10, M-11, M-18, and M-20) on TER measurement and immunofluorescence for ZO-1 as a marker of the tight junction.

MATERIALS AND METHODS

Chemicals. Nefiracetam and its five main metabolites (M-3, M-10, M-11, M-18, and M-20) were synthesized at Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), and cytochalasin-B, a cytotoxicant (Ma et al., 1995; Nybom and Magnusson, 1996), was purchased from Sigma (St. Louis, MO). Other reagents utilized were of the best analytical grade.

Animals and housing conditions. Male beagle dogs who were 31 months of age and purchased from Toyota Tsusho Corporation (Tokyo, Japan) were used for the investigation. They were individually housed at an environmental temperature of 23 ± 2°C and a relative humidity of 60 ± 20% with a 12 h light/dark cycle. The animals were allowed access to a commercial laboratory diet (DS, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. All dogs were treated humanely, and the study protocol was in accordance with the Institutional Guidelines of Daiichi Pharmaceutical Co., Ltd. for use of laboratory animals.

Isolation of uroepithelial cells of the urinary bladder. Dogs from a control group used in a toxicological study were killed by exsanguination under sodium pentobarbital anesthesia (25 mg/kg, iv; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), and the bladder was aseptically excised. An incision was then made lengthwise along the bladder, and the opened bladder was washed three times with the Krebs solution (110 mM NaCl, 5.8 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 2.0 mM CaCl₂, 1.2 mM MgSO₄, and 11.1 mM glucose, pH 7.4) at 4°C. Afterward, the bladder was trimmed to remove excess fatty tissues and transferred, mucosal side down, to a metal rack with 10 sharp metal pins along each edge (Lewis and Hanrahan, 1990), which was placed in the same solution at 4°C, and the smooth muscle layer was carefully removed. The tissue was stretched, mucosal side up, across the metal pins on a 10 × 10-cm square plate, and then incubated for 24 h at 4°C in the minimum essential medium (MEM; Life Technologies Inc., Grand Island, NY) containing 1% (v/v) penicillin/streptomycin/fungizon (PSF; Life Technologies Inc.), 2.5 mg/ml dispase (Life Technologies Inc.), and 20 mM HEPES (MEM/PSF/ dispase solution, pH 7.4).
After incubation, the MEM/PSF/dispace solution was aspirated, the stripped mucosa was transferred to a sterile 150-mm culture dish, and uroepithelial cells were scraped from the connective tissues with cell scrapers. The scraped cells were suspended in 20 ml of trypsin-EDTA (0.25% trypsin and 1 mM EDTA - 4Na. Life Technologies Inc.), and incubated for 30 min at 37°C. Later, the single cell suspension was brought up to 50 ml with MEM containing 1% PSF, 5% fetal bovine serum (PBS; Life Technologies Inc.) and 20 mM HEPES (MEM/PSF/FBS solution, pH 7.4) in a sterile tube and spun down with a centrifuge (KUBOTA 8800, KUBOTA Corporation, Tokyo, Japan) at 1000 rpm for 5 min at 4°C. The resulting supernatant was aspirated carefully and the cells were suspended in 50 ml of the same MEM/PSF/FBS solution. This washing process was repeated two more times. The cells were then rewarshed in 50 ml of the keratinocyte medium (defined keratinocyte-SFM, Life Technologies Inc.) and resuspended in the appropriate volume of the keratinocyte medium to make a final concentration of 6.0–7.0 × 10^5 cells/ml, as determined by counting the cells in a hemocytometer chamber.

**Cell culture.** The collagen solution was prepared by mixing 5 mg type IV collagen (Sigma), 100 μl glacial acetic acid, and 50 ml distilled water, and kept overnight at 4°C without stirring. The collagen solution was sterilized with a 0.22-μm bottle top filter (Asahi Techno Grass Corporation, Tokyo, Japan) and stored at 4°C. The keratinocyte medium was added to both chambers with a 12-mm transwell filter (Corning Coaster Corporation, Cambridge, MA) and incubated for 2 h at 37°C. Prior to use, the collagen solution was diluted 1:9 with 10 mM Na2CO3-HCl (pH 9.0), and 500 μl of the resultant solution was added to each apical chamber after aspirating the keratinocyte medium and incubated for 1 h at 37°C.

Before plating, the collagen solution was aspirated, 0.5 ml of the cell suspension was added to the apical chamber, 1.5 ml of keratinocyte medium was added to the basal chamber, and cells were incubated at 37°C. Three days later, the apical and basal media were aspirated and replaced with 0.5 and 1.5 ml of the keratinocyte medium containing 1 mM CaCl2 (KM/Ca solution), respectively, in case the TER reached levels of approximately 1000 ohm-cm^2 or higher.

**TER measurement.** TER was measured using an epithelial voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL). The electrodes were sterilized by immersing them in 70% ethanol, and they were washed with sterile PBS prior to use. Calculations for ohm-cm^2 were made by subtracting values of blank inserts from all samples and multiplying by the area seeded with cells.

**Light and electron microscopy.** Cells cultured on a 12-mm transwell filter were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), rinsed with 8.2% sucrose, postfixed in 1% OsO4 in the buffer, dehydrated through an ascending alcohol series, and embedded in epoxy resin. Semi-thin sections were prepared and stained with uranyl acetate and lead citrate, and ultrathin sections were stained with toluidine blue for light microscopic examination. Ultrastructural examination of cultured uroepithelial cells showed that TER started to increase from day 3 onward, and reached a maximum value on day 7 or 8. Then, TER tended to decrease gradually and disappeared by day 20 (Fig. 2). Light and electron microscopic appearances, the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A), and the cultured cells consisted of three distinct cell layers (superficial, intermediate, and under cell layers, Fig. 3A). Immunofluorescence observations for ZO-1 revealed that treatment with cytochalasin-B resulted in a slight disappearance of the fluorescence band around the cells at 4 μM, and caused a disruption and spotty appearance of the fluorescence band along the cell border, and finally cell disappearance at 10 μM (Fig. 4B). No remarkable effects were noted at 1.6 μM.
which was due to large interindividual variations. No significant reduction was observed, though not statistically significant, 48 h later, and at 2 mM, TER was significantly decreased from M-18 at 0.8 mM caused a significant reduction of TER from the urinary bladder epithelium (Truschel, 1990). More recently, the primary uroepithelial culture system of the rabbit urinary bladder has been reported to be an appropriate tool for assessing the barrier function and morphology of the urinary bladder such as high TER and three cell layers (Denker and Nigam, 1998). Our results demonstrated that the culture system possessed functional and morphological characteristics of uroepithelial cells of the urinary bladder such as high TER and three cell layers with junctional complex.

Effect of Nefiracetam and Its Metabolites on TER and Immunofluorescence for ZO-1

Effects of nefiracetam and its five metabolites on TER of the cultured uroepithelial cells were investigated. Cells with a high TER (over 10,000 ohm-cm², about four or five days after plating) were used for examination. In TER measurements, M-18 at 0.8 mM caused a significant reduction of TER from 48 h later, and at 2 mM, TER was significantly decreased from 24 h later, except for 120 h later. At 120 h, the persistent reduction was observed, though not statistically significant, which was due to large interindividual variations. No significant changes in TER were observed in the other groups throughout the observation period (Fig. 5). Cytochalasin-B at 10 μM used as a positive control showed decreased TER from 1 h as in the aforementioned study. In immunofluorescence appearances, both M-10 and M-18 exhibited slight deformation of the uroepithelial cells and a slight reduction of ZO-1 band 120 h later (data not shown).

DISCUSSION

Although numerous attempts have been made to develop cell culture models of the uroepithelium, few systems have possessed physiological properties of the uroepithelium reflected in vivo (Chlapowski and Haynes, 1979; Guhe and Follmann, 1994; Howlett et al., 1986; Perrone et al., 1996; Surya et al., 1990). More recently, the primary uroepithelial culture system of the rabbit urinary bladder has been reported to be an appropriate tool for assessing the barrier function and morphology of the urinary bladder epithelium (Truschel et al., 1999). Therefore, we tried to reproduce urinary bladder lesions seen in dogs using rabbits in various regimens so that the rabbit uroepithelial culture system was available. Unfortunately, nefiracetam showed neither similar lesions in rabbits in vivo nor effects on the primary uroepithelial cells of the rabbit urinary bladder in vitro (unpublished data). On the basis of this information, to delineate the pathogenesis of urinary bladder lesions in dogs due to nefiracetam, we developed the primary uroepithelial culture of the canine urinary bladder, and then explored the effects of nefiracetam and its main metabolites on the cultured uroepithelial cells.

First of all, we evaluated time-course changes in TER and morphology of cultured uroepithelial cells to substantiate the barrier function in the naive state. The epithelium of the urinary bladder is shown to have one of the highest TERs in the whole body with values ranging from 10,000 to 75,000 ohm-cm² (Lewis and Diamond, 1976). In the present study, as a device to prevent keratinocyte stratification and differentiation, the medium was formulated to have a low calcium concentration (<0.1 mM). TER was monitored during the 20-day observation periods. On day 3, TER achieved 1000–3000 ohm-cm², in which values were high as compared with those of rabbit cultured uroepithelial cells (about 200 ohm-cm², Truschel et al., 1999). When TER was over 1000–3000 ohm-cm², the culture medium was replaced with the keratinocyte medium containing 1 mM CaCl₂. In a case that uroepithelial cells were grown in the medium without 1 mM CaCl₂, or the medium replacement was delayed by only one day, uroepithelial cells failed to achieve high TER. From days 5 to 10, TER reached 16,000 ohm-cm² or more, suggesting that the canine uroepithelial cells have the tight junction, since high TER emerged as the feature of culture cells forming a tight junction-like function to ion flux (Schneeberger and Lynch, 1992).

Morphologically, the uroepithelium of the urinary bladder comprised three cell layers with a superficial cell layer consisting of cells forming junctional complex (containing tight and adherence junctions). Furthermore, the immunofluorescence for both ZO-1 and E-cadherin was observed around the respective cultured cells. ZO-1 is an essential protein associated with the tight junction, and E-cadherin is necessary to form the adherence junction (Denker and Nigam, 1998). Our results demonstrated that the culture system possessed functional and morphological characteristics of uroepithelial cells of the urinary bladder such as high TER and three cell layers with junctional complex.

Cytochalasin-B induced decreases in TER, disruption of actin microfilaments, and marked increases in paracellular permeability (Ma et al., 1995). The decrease in TER with the disruption of ZO-1 band is thought to be due to an alteration of the actin structure, because actin microfilaments closely correlate with the tight junction and the localization of ZO-1 proteins. In addition, since actin microfilaments and microtubules play a critical role in cytoskeletal formation, it is proposed that depolymerization of these elements may be related to cell deformation. Hence, effects of cytochalasin-B on TER and immunofluorescence for ZO-1 were examined to validate the culture system developed. Cytochalasin-B decreased TER from 1.6 μM in a concentration-dependent manner. In the
immunofluorescence, this compound displayed induced a slight reduction of the ZO-1 band at 4 μM and a disruption of ZO-1 band and cell disappearance at 10 μM. These findings indicate that the culture system can be successfully performed and evaluate disturbed tight junction arising from the new chemical entity.

Among nefiracetam and its five main metabolites employed, only M-18 elicited a significant decrease in TER from 0.8 mM. In immunofluorescence appearances, M-18 exhibited a slight reduction of ZO-1 band and deformation of the uroepithelial cells. Meanwhile, M-10, a monohydroxylated derivative of nefiracetam with a sulfate-conjugating metabolite of M-18,

FIG. 3. Morphological findings of the primary uroepithelial cells of the canine urinary bladder. Cells were cultured in the keratinocyte medium containing 1 mM CaCl₂ on a 12-mm transwell filter for seven days. (A) Microscopic appearance. The cultured cells consisted of three layers. S, superficial cell layer; I, intermediate cell layer; U, under cell layer. Original magnification, ×200. (B) Ultrastructural appearance. TJ, tight junction. Original magnification ×40,000. (C) Confocal immunofluorescence. (a) ZO-1, (b) E-cadherin. Cells were incubated in the keratinocyte medium containing 1 mM CaCl₂ on a 12-mm transwell filter for 10 days. Original magnification ×630.
also induced deformation of the uroepithelial cells in the late phase (120 h later). This implied that the effect of M-18 on uroepithelial cells was stronger than that of M-10. M-18 is reported to show a high concentration in canine serum as compared with other species (unpublished data). Because the measurement of M-18 in urine has been extremely difficult, its quantitative data have not been procured. The possibility is raised that a high concentration of M-18 is present in urine as well as in serum. According to the previous report from Kashida et al. (1996), it was suggested that the direct action of nefiracetam or its metabolites in urine induced degeneration of epithelial cells of the urinary bladder. On the other hand, it is reported that cyclophosphamide, a typical toxicant of the urinary bladder, caused hemorrhagic cystitis, preceded by contact with acrolein, a metabolite of cyclophosphamide (Fraiser and Kehrer, 1992; Pohl et al., 1989). Likewise, cystitis has been confirmed to be brought about by the direct toxicity of a carbonic anhydrase inhibitor (Durand-Cavagna et al., 1992) or trimethyl imidazopyrazolopyrimidine (Macallum and Albasam, 1994) to the urinary bladder wall. Their mechanisms remain unclear, but it is speculated that their metabolites excreted into urine play an important role. In urinalyses from a previous dog study, although increases in protein excretion and positive occult blood were observed from two-week oral treatment of nefiracetam, neither the fluctuation of urinary pH and osmotic pressure nor the presence of medicine-like crystals was noted (unpublished data). In rats and monkeys, no effects of nefiracetam on urinalyses were seen (unpublished data). In the present study, pH and osmotic pressure in the culture medium containing nefiracetam, M-10, and M-18 at 2 mM were similar to those in the control medium. Further, M-18

FIG. 4. Effect of cytochalasin-B on primary uroepithelial cells of the canine urinary bladder. (A) Transepithelial electrical resistance (TER) in the cells exposed to cytochalasin-B at 1.6, 4, and 10 μM was measured 1, 2, 4, 8, 24, and 48 h later. Vehicle control, circle; 1.6 μM, triangle; 4 μM, square; 10 μM, diamond. Data are shown as the mean ± SD (n = 6). Significant difference from the vehicle control group (**p < 0.01). (B) Immunofluorescence for ZO-1 in the cells exposed to cytochalasin-B at 10 μM 48 h later. Cytochalasin-B induced a disruption of the ZO-1 band, spotty appearance of ZO-1 protein along the uroepithelial cells (arrow head) and cell disappearance (arrow). Original magnification ×630.

FIG. 5. Transepithelial electrical resistance (TER) in primary uroepithelial cells of the canine urinary bladder exposed to nefiracetam and its metabolites at 0.8 or 2 mM. Vehicle control, filled circle; nefiracetam, triangle; M-3, square; M-10, diamond; M-18, open circle. TER was measured 24, 48, 72, and 120 h later. Data are shown as the mean ± SD (n = 5–6; n = 3 only for M-3). Significant difference from the vehicle control group (*p < 0.05; **p < 0.01).
showed no cytotoxic effect on Mardin-Darby canine kidney cells or canine renal papilla slices at a concentration of up to 5 mM in a previous in vitro study (unpublished data). Taken together, M-18 in urine may cause alterations of actin microfilaments and microtubules leading to cell deformation in the uroepithelium of the canine urinary bladder, preceded by reductions in TER and ZO-1 band.

In conclusion, the canine uroepithelial culture system possessed both functional and morphological features of the uroepithelium reflected in vivo. In the urinary bladder lesion in dogs due to nefiracetam, the metabolite M-18 may strongly contribute to the process of its occurrence.

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