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Involvement of cyclooxygenase-2 and EP_3 prostaglandin receptor in acute herpetic but not postherpetic pain in mice

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Abstract

The precise mechanisms of zoster-associated pain and postherpetic neuralgia remain unknown. Inoculation of mice with herpes simplex virus type-1 elicits acute herpetic pain- and delayed postherpetic pain-related responses. We investigated the role of prostaglandins (PGs) and their synthases in both types of pain. Deficiency in EP_3 but not EP_1, IP or TP prostanoid receptor markedly diminished the acute herpetic pain and resulted in the decrease of the incidence of the delayed postherpetic pain. Preventive but not therapeutic administration of the EP_3 antagonist ONO-AE3-240 inhibited the acute herpetic pain. The non-selective cyclooxygenase (COX) inhibitor diclofenac and the selective COX-2 inhibitors NS-398 and JTE-522 dose dependently reduced the acute herpetic pain, and NS-398 was without effect on delayed postherpetic pain. COX-2 was induced and PGE_2 content was increased in the affected dorsal root ganglia at the stage of acute herpetic pain. COX-2-like immunoreactivities were found around the nuclear membrane of many dorsal root ganglion neurons that were negative for herpesvirus antigen. COX-2 mRNA expression and PGE_2 content in the affected dorsal root ganglia at the stage of delayed postherpetic pain were similar to those of naive mice. The propagation of herpes virus in dorsal root ganglion may induce COX-2 and produce PGE_2 in uninfected neurons. The results suggest the important roles of COX-2 induction and the PGE_2–EP_3 receptor system in the dorsal root ganglia in the development but not maintenance of acute herpetic pain. It was further confirmed that the PG systems do not play a key role in delayed postherpetic pain.

Keywords: Acute herpetic pain; Delayed postherpetic pain; Allodynia; Hyperalgesia; Cyclooxygenase; Prostaglandin

1. Introduction

Herpes zoster, characterized by a dermal rash and severe pain, is caused by the reactivation of dormant human herpesvirus 3 (varicella-zoster virus) in the sensory ganglion in human subjects (Loeser, 1986). Patients with herpes zoster complain of severe spontaneous pain, allodynia (pain resulting from innocuous stimulation), and hyperalgesia (abnormally increased pain induced by noxious stimulation). In some herpes zoster patients, pain persists for more than 3–6 months after healing of the skin lesions, which is termed postherpetic neuralgia (Dworkin and Portenoy, 1996).

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Patients with postherpetic neuralgia report various types of pain, including a continuous burning or aching pain, a periodic piecing pain, and allodynia (Dworkin and Portenoy, 1996). Once established, postherpetic neuralgia is particularly difficult to treat and is often resistant to conventional analgesics. At present, the precise mechanisms of acute herpetic pain and postherpetic neuralgia are unclear.

Varicella-zoster virus infection into the rat hind paw causes long-lasting allodynia and hyperalgesia in rats (Fleetwood-Walker et al., 1999). This is an interesting animal model of postherpetic neuralgia, but there is no herpetic pain stage. A mode of varicella-zoster virus infection is different between humans and animals and the inoculation does not produce herpes zoster in animals. On the other hand, although the reactivation of dormant human herpesvirus 1 (herpes simplex virus type-1, HSV-1) in the sensory ganglion results in herpes simplex in humans, a transdermal inoculation of HSV-1 causes herpes zoster-like skin lesions in mice (Takasaki et al., 2000a). This may be due to a similarity in the mode of propagation between varicella-zoster virus in humans and HSV-1 in animals. In humans, latent varicella-zoster virus is found in the satellite cells that encircle many sensory neurons and reactivated viruses spread to many surrounding neurons. On the other hand, latent HSV-1 is found in sensory neurons and its reactivation results in the localized vesicular eruption, suggesting that this herpesvirus does not spread to many sensory neurons. Patients with herpes simplex generally complain of localized pain and some patients complain of neuralgia similar to postherpetic neuralgia (Gonzales, 1992).

When HSV-1 is inoculated on the skin of the mouse, it proliferates in many (about 20%) sensory neurons in the dorsal root ganglion (DRG) and herpes zoster-like skin lesions develop in the inoculated dermatome from day 5 after inoculation (Takasaki et al., 2000a,b). They show pain-related responses to innocuous tactile and noxious mechanical stimulation (tactile allodynia and mechanical hyperalgesia, respectively) (Takasaki et al., 2000a), without thermal hyperalgesia (Sasaki et al., 2003). DNA of HSV-1 is increased in the DRGs at L4–5 levels on the inoculated side from 3 to 5 days after inoculation and decreased by day 6 after inoculation (Takasaki et al., 2000a). HSV antigen-like immunoreactivity was observed in the corresponding DRGs on day 5 after inoculation and was markedly decreased on day 7 (Takasaki et al., 2000b). These findings suggest that inoculated HSV-1 infects primary afferents, passes along sensory nerves, and is replicated in the DRG. Zoster-like lesions and pain-related responses may be due to the virus propagation in the DRG. Zoster-like lesions completely heal by day 15 after inoculation (Takasaki et al., 2002). Interestingly, about half of the mice with acute herpetic pain show pain-related responses long after the lesions completely heal, suggesting the development of postherpetic pain in mice (Takasaki et al., 2002; Kuraishi et al., 2004).

In the above murine models, the non-steroidal antiinflammatory drug diclofenac inhibits the acute phase of pain-related responses, but it does not affect postherpetic pain-related responses (Takasaki et al., 2000b, 2002). These findings suggest the involvement of prostanoid(s) in herpetic, but not postherpetic, pain-related responses. Prostanoids are a group of bioactive lipids working as local mediators and are comprised of D, E, F, and I types of prostaglandins (PGs) and thromboxane. Prostanoids exert various pathophysiological effects via a variety of seven-transmembrane domain receptors. Prostanoid receptors have been cloned and designated DP, EP (EP_1, EP_2, EP_3, and EP_4), FP, IP, and TP (Narumiya et al., 1999). PGs including PGE_2 and PGI_2, oxygenated metabolites of arachidonic acid are produced by the sequential actions of cyclooxygenase (COX) and specific synthases. Two isoforms of COX have been identified; COX-1 is constitutively expressed in various tissues, whereas COX-2 is induced by mitogens and cytokines. In this study, we examined the differences in the content of prostanoids and COX between mice with acute herpetic and postherpetic pain. We also investigated the roles of prostanoid receptors in the acute herpetic pain.

2. Methods

2.1. Animals

Female BALB/c mice (6 weeks old at the start of experiment; Japan SLC, Shizuoka, Japan) were generally used. In a series of experiments, male C57BL/6J mice (Japan SLC) and prostanoid receptor gene-deficient mice ($EP_1^{-/-}$, $EP_3^{-/-}$, $IP^{-/-}$, and $TP^{-/-}$) with genetic background of C57BL/6J were used. Experiments were conducted with the approval of the Animal Care Committee at Toyama Medical and Pharmaceutical University, and according to the guidelines for investigations of experimental pain in animals published by the International Association for the Study of Pain (Zimmermann, 1983).

2.2. HSV-1 inoculation

Mice were inoculated with HSV-1 as described previously (Takasaki et al., 2000a). In brief, HSV-1 (7401H strain, 1×10^6 plaque-forming units in 10 μl) was inoculated on the shin skin of the right hind paw (5×5 mm) after scarification with 27-gauge needles. The contralateral hind paw was without inoculation.

Skin lesions observed on the inoculated side were scored as follows: 0, no lesions; 2, one or two vesicles on

the back; 4, many vesicles on the back and/or surrounding inoculated area; 6, mild herpes zoster-like lesions; 8, apparent zoster-like lesions and/or paw inflammation; 10, severe zoster-like lesions (Takasaki et al., 2000a).

2.3. Assessment of pain-related responses

Pain-related responses of the hind paw were assessed using von Frey filament (VFF), as described previously (Takasaki et al., 2000a). After at least 15-min acclimation period, VFFs with bending force of 0.17 or 1.20 g were pressed perpendicularly against the plantar skin of the hind paw and held for 3–5 s with it slightly bent. The responses to these stimuli were ranked as follows: 0, no response; 1, move away from VFF; 2, immediate flinching or licking of the hind paw. The stimulation of the same intensity was applied six times to each hind paw at intervals of several seconds. In accordance with a previous report (Kuraishi et al., 2004), allodynia and hyperalgesia were determined as follows: since all normal mice tested do not respond to VFF of 0.17-g strength, mice that show 0.5 or higher response scores were considered to have allodynia. When stimulated with VFF of 1.20-g strength, naive mice showed 0.5 or lower response scores. Thus, in this case, mice that show 1.17 or higher response scores were considered to have hyperalgesia.

Analgesic effect was calculated as follows:

$$\text{Analgesic effect (\%)} = \frac{\text{PS (inoculated, before drug)} - \text{PS (inoculated, after drug)}}{\text{PS (inoculated, before drug)} - \text{PS (contralateral, before drug)}} \times 100$$

where PS is pain-related score.

2.4. Agents

The non-steroidal antiinflammatory drug/non-selective COX inhibitor diclofenac sodium (Research Biochemical International, Natick, MA) and the COX-2 inhibitor NS-398 (*N*-(2-[cyclohexyloxy]-4-nitrophenyl)-methanesulfonamide; Sigma, St. Louis, MO) were resolved in physiological saline and 20% dimethylsulfoxide, respectively. They were administered intraperitoneally. Another COX-2 inhibitor JTE-522 (4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonamide; a gift from Japan Tobacco Inc., Tokyo, Japan) was suspended in 0.5% carboxymethylcellulose and administered orally. The selective EP_3 antagonist ONO-AE3-240 (a gift from Ono Pharmaceutical Co. Ltd., Osaka, Japan) (Amano et al., 2002) was suspended in 0.5% methylcellulose and injected subcutaneously. To prevent motor paralysis and death, BALB/c mice were given acyclovir orally (10 mg/kg) five times (09:00, 12:00, 15:00, 18:00, and 21:00 h) daily from day 5 to 11 after inoculation; this dosing completely prevented the motor paralysis and death without effects on the skin lesions, acute pain-related behaviors, and body weight (Takasaki et al., 2002).

2.5. Preparation of dorsal root ganglion (DRG)

After decapitation under diethyl ether anesthesia, the DRGs on both sides at the L4 and L5 levels were rapidly removed. For the determination of mRNA and PG contents, the DRG preparations were stored at −80 °C until assay. For immunohistochemistry, the DRGs were frozen in OCT compound on dry ice and stored at −80 °C until cryostat sectioning.

2.6. RT-PCR and southern blot hybridization

Transcripts encoding COX-1, COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by RT-PCR followed by southern blot hybridization. Total RNA was extracted from the DRG preparation, and cDNA was synthesized from total RNA using an oligo $(dT)_{16}$ primer and Superscript™ II (GIBCO BRL, Grand Island, NY) in a 20-μl reaction mixture. cDNA (1 μl) was amplified with 1 U of Taq DNA polymerase in a 20-μl reaction mixture. The amplification protocol comprised 30 cycles (COX-1), 39 cycles (COX-2) or 24 cycles (GAPDH) of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. Primers used (Hokkaido System Science Co. Ltd., Sapporo, Japan) were as follows: 5′-ACC ACT CGC CTC ATC CTT AT-3′ (sense) and 5′-GCA CAC GGA AGG AAA CAT AG-3′ (antisense) for COX-1; 5′-ATC CAC AGC CTA CAA AAA CAG-3′ (sense) and 5′-AAC CTC ACA GCA AAA ACC TAC-3′ (antisense) for COX-2; 5′-CAA AGG TCA TCC ATG ACA AC-3′ (sense) and 5′-TTA CTC CTT GGA GGC CAT GT-3′ (antisense) for GAPDH. Reaction mixtures were separated on a 1.5% agarose gel. Southern blot hybridization was performed using 40-mer 5′-end digoxigenin-labeled probes (Greiner Japan, Tokyo, Japan): 5′-ACC TCT TTC GGT ATT CAT TGA AGG GCT GTA GGC GCA TCT C-3′ for COX-1; 5′-AGC TGT TTA CTC CAT GTC GGT ACA AAC CTG ACA GCT TAA G-3′ for COX-2; 5′-TTG AAG TCG CAG GAG ACA ACC TGG TCC TCA GTG TAG AAA A-3′ for

GAPDH. To determine the expression levels of COX-1, COX-2, and, GAPDH mRNAs, we measured the density of the bands with a densitometer (DensitoGraph, ATTO, Tokyo, Japan).

2.7. Determination of PG contents

The DRG samples were homogenized in ethanol containing 0.1 μM indomethacin. After centrifugation, the supernatant was applied to a Sep-Pak C18 column and PGs were then eluted with diethyl ether. The eluate was dried, and the residue containing PGE_2 and 6-keto $PGF_{1\alpha}$ (a stable metabolite of PGI_2) were assayed with respective enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI).

2.8. Fluorescence immunohistochemistry

DRGs were sectioned at 10 μm in thickness with a cryostat and sections were mounted on glass slides. The sections were fixed with 4% paraformaldehyde for 30 min and washed three times in phosphate-buffered saline (PBS). They were incubated in PBS containing 1.5% fetal bovine serum at room temperature for 30 min and then with goat anti-COX-2 antiserum (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rabbit anti-HSV-1 antiserum (1:100; DAKO, Copenhagen, Denmark) overnight at 4 °C in a humid chamber. After incubation with the primary antibodies, they were washed with PBS, and incubated with biotin-labeled donkey anti-goat IgG (1:100) (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. They were then rinsed with PBS, and incubated with fluorescein isothiocyanate-labeled avidin (1:1,000) (Vector Laboratories Inc., Burlingame, CA) and Cy3-labeled donkey anti-rabbit IgG (1:400) (Chemicon International Inc., Temecula, CA), and coverslipped with glycerol-PBS containing 2.5% triethylenediamine. The sections were observed with a confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss Co. Ltd., Jena, Germany).

2.9. Data analysis

The means of data are presented together with SEM. Data were analyzed with one-way analysis of variance (ANOVA), Friedman repeated measures ANOVA on ranks or Kruskal–Wallis one-way ANOVA on ranks. Statistical differences between groups were analyzed with Dunnett's multiple comparisons or chi-square test. $P<0.05$ was considered significant.

3. Results

3.1. Induction of acute herpetic pain and delayed postherpetic pain

The transdermal inoculation of mice (BALB/c strain) with HSV-1 developed herpes zoster-like skin lesions in all animals tested. Although the stimulation of the hind paw of naive mice with von Frey filament (VFF) of 0.17-g strength did not produce any behavioral responses, the stimulation of the inoculated paw elicited aversive responses (tactile allodynia) from day 5 after inoculation (Fig. 1A). The stimulation with 1.20-g VFF elicited aversive responses in naive mice, and an increase in the responses (mechanical hyperalgesia) of the inoculated hind paw became apparent from day 5 (Fig. 1B). The allodynia and hyperalgesia were relatively constant from day 6 to day 8 after inoculation.

When BALB/c mice were inoculated with HSV-1 and given acyclovir (10 mg/kg five times a day) from day 5 to 11 after inoculation, all skin lesions completely healed by day 15 after inoculation. On day 30 after inoculation, mice were classified into two groups; positive or negative for delayed postherpetic pain-related responses (allodynia and hyperalgesia), according to our previous report (Kuraishi et al., 2004). Although 18 of 40 mice showed

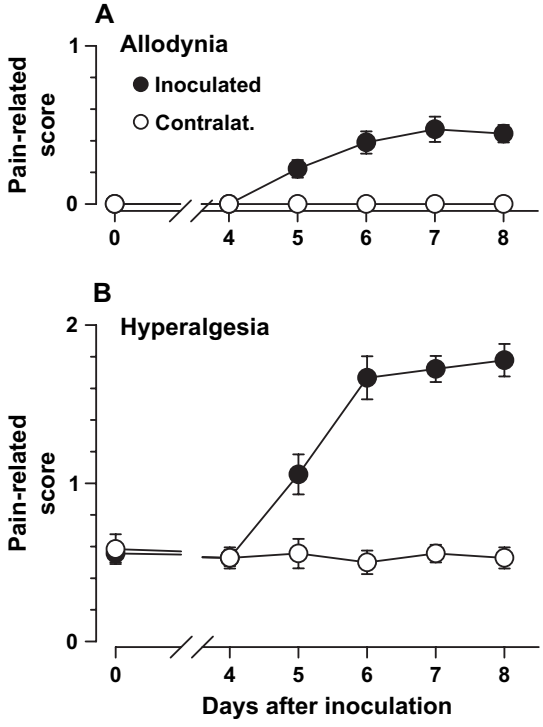

Fig. 1. Effects of HSV-1 inoculation on pain-related responses in BALB/c mice. The mice were inoculated with HSV-1 on the right hind paw with the contralateral hind paw untreated. Allodynia (A) and hyperalgesia (B) were assessed. The data presented are means and SEM ($n=6$).

no allodynia and hyperalgesia, the rest (22 of 40 mice) showed marked pain-related responses (Table 1).

HSV-1 inoculation also induced herpes zoster-like skin lesions in C57BL/6J mice, but the degree of skin lesions was significantly (Dunnett's test) milder in C57BL/6J mice than in BALB/c mice. Unlike BALB/c strain, skin lesions were completely healed by day 15 after inoculation without acyclovir treatment. There were no significant differences (Dunnett's test) in the acute herpetic pain-related scores between BALB/c and C57BL/6J mice. The incidence (63%, 45 of 71 mice) of delayed postherpetic pain of C57BL/6J mice was significantly (chi-square test) higher than that (45%, 18 of 40 mice) of BALB/c mice. Pain-related response to 1.20-g VFF of C57BL/6 mice with postherpetic pain was significantly higher (Dunnett's test) than that of BALB/c mice with postherpetic pain (Table 1). There were increased tendencies of pain-related score at the acute phase (on day 6) in mice with delayed postherpetic pain of either strain (Table 1).

3.2. Effects of prostanoid receptor deficiencies on acute herpetic pain

To determine the subtypes of prostanoid receptors involved in the acute herpetic pain, we used mice deficient in prostanoid receptor gene. In control mice of C57BL/6J strain, HSV-1 inoculation produced allodynia and hyperalgesia from day 5 after inoculation (Fig. 2A). In $EP_3^{-/-}$ mice, allodynia and hyperalgesia were not evident on days 5 and 6 after inoculation. The pain-related responses became apparent from day 7 after inoculation, but the degree was markedly smaller than that of the control mice (Fig. 2A). The degree of peak allodynia and hyperalgesia was not significantly different (Dunnett's test) between $EP_1^{-/-}$, $IP^{-/-}$, and $TP^{-/-}$ and control mice (Fig. 2A,B). HSV-1 inoculation produced herpes zoster-like skin lesions in all mice tested, but the degree of skin lesion was not significantly different (Dunnett's test) between $EP_1^{-/-}$, $EP_3^{-/-}$, $IP^{-/-}$, and $TP^{-/-}$ and control mice (data not shown). The incidence of delayed postherpetic pain was significantly (chi-square test) lower in $EP_3^{-/-}$ mice than in control C57BL/6J mice; 2 (15%) of 13 $EP_3^{-/-}$ mice and 9 (64%) of 14 C57BL/6J mice had delayed postherpetic pain.

3.3. Effects of EP_3 receptor antagonist

When injected on day 6 after inoculation, the EP_3 receptor antagonist ONO-AE3-240 at subcutaneous doses of 3–30 mg/kg did not affect the acute herpetic pain-related responses ($n=6$ each group, data not shown). We did not examine the higher doses because of difficulty in preparing a suspension. When ONO-AE3-240 (30 mg/kg) was injected three times daily from day of viral inoculation, it significantly (Friedman repeated measures ANOVA on ranks) inhibited the development of allodynia and hyperalgesia without effects on the responses of contralateral hind paw (Fig. 3).

3.4. Effects of COX inhibitors

When injected on day 6 after inoculation, diclofenac sodium at intraperitoneal doses of 10–100 mg/kg dose dependently (Kruskal–Wallis one-way ANOVA on ranks) attenuated both allodynia and hyperalgesia; however, the inhibitions of hyperalgesia after doses of 30 and 100 mg/kg were incomplete and similar to each other (Fig. 4A). The selective COX-2 inhibitor JTE-522 at oral doses of 1–10 mg/kg produced dose-dependent (Kruskal–Wallis one-way ANOVA on ranks) inhibition of allodynia and hyperalgesia (Fig. 4B). Another selective COX-2 inhibitor NS-398 at intraperitoneal doses of 5 and 10 mg/kg also produced dose-dependent (Kruskal–Wallis one-way ANOVA on ranks) inhibition (Fig. 4C). NS-398 at a dose of 10 mg/kg exerted no apparent influences on allodynia and hyperalgesia on day 35 after inoculation ($n=8$ for NS-398 and vehicle groups, data not shown).

Table 1
Induction of delayed postherpetic pain (DPP) in BALB/c and C57BL/6J mice

Strain	DPP	n (%)	Skin lesion score	Pain-related score			
				0.17-g VFF		1.20-g VFF	
				Day 6	Day 30	Day 6	Day 30
BALB/c	+	18 (45)	7.8±0.7	0.54±0.06	0.53±0.04	1.51±0.08	1.42±0.04
	−	22 (55)	7.7±0.6	0.43±0.05	0.03±0.02	1.38±0.05	0.58±0.04
C57BL/6J	+	45 (63)[a]	6.5±0.2[b]	0.56±0.03	0.58±0.03	1.60±0.04	1.61±0.03[b]
	−	26 (37)	6.8±0.4[b]	0.47±0.03	0.00±0.00	1.50±0.05	0.59±0.03

Data of skin lesion score and pain-related responses are expressed as mean±SEM. Skin lesions were scored on day 7 after viral inoculation and pain-related responses to von Frey filaments (VFF) of 0.17- or 1.20-g strength were assessed on days 6 and 30; see Section 2 for scoring. Mice that had allodynia or hyperalgesia on day 30 were considered positive for DPP and all mice were classified into DPP-positive and negative groups. BALB/c mice were given acyclovir from day 5 to 11 to prevent motor paralysis and death.

[a] The incidence of DPP was significantly higher than that of BALB/c mice ($P<0.05$, chi-square test; odds ratio=5.29).

[b] $P<0.05$ as compared with scores in BALB/c strain (Dunnett's test).

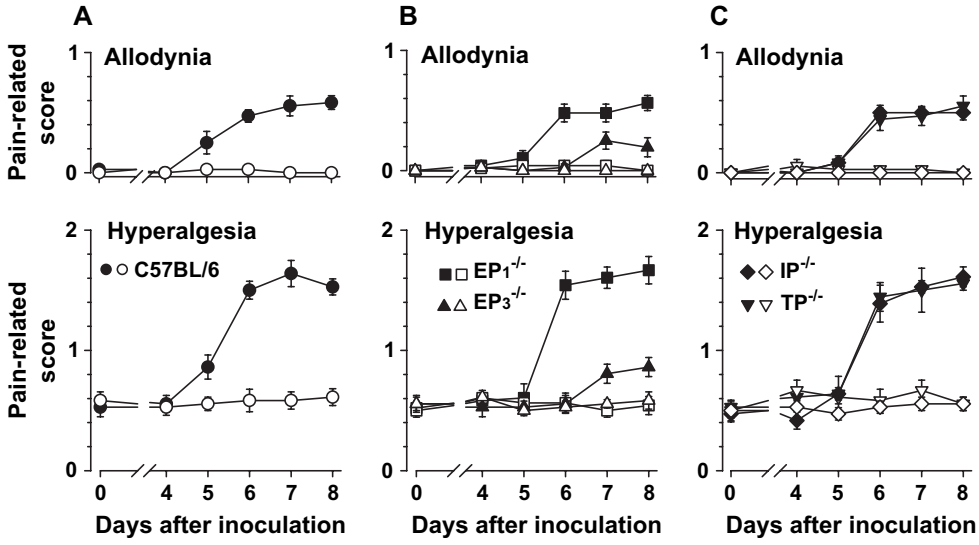

Fig. 2. Effects of deficiencies in prostanoid receptor subtypes on HSV-1 inoculation-induced pain-related responses. (A) Effects of HSV-1 inoculation on pain-related responses in C57BL/6J mice. (B) Effects of EP_1 and EP_3 receptor deficiencies. (C) Effects of IP and TP receptor deficiencies. HSV-1 was inoculated on the unilateral hind paw of C57BL/6J ($n=6$), $EP_1^{-/-}$ ($n=8$), $EP_3^{-/-}$ ($n=6$), $IP^{-/-}$ ($n=6$), $TP^{-/-}$ mice ($n=6$). Allodynia (upper panels) and hyperalgesia (lower panels) were tested. Closed symbols, inoculated side; open symbols, the contralateral side. Each point represents means and SEM. Allodynia and hyperalgesia were significantly different between control and $EP_3^{-/-}$ mice during a period from day 4 to day 8 post-inoculation (Friedman repeated measures ANOVA on ranks).

3.5. Effects of HSV-1 infection on PG contents and COX levels in DRG

HSV-1 inoculation markedly increased the content of PGE_2 in the DRG (L4 and L5 levels) on the inoculated side on days 5 and 7 after inoculation, although it was not altered on day 3 and returned to the normal level on day 30 (Fig. 5A). HSV-1 inoculation did not affect the content of 6-keto $PGF_{1\alpha}$ (a stable metabolite of PGI_2) at all time points tested (Fig. 5B).

The expression level of COX-1 mRNA in the DRG was not altered after HSV-1 inoculation (Fig. 5C). Although the level of COX-2 mRNA in the DRG of naive mice was as low as the detection limit and not altered on day 3 after inoculation, it was markedly increased on the inoculated side on days 5 and 7 after inoculation (Fig. 5D). The level of COX-2 mRNA in the contralateral DRG was slightly but significantly increased on day 5 after inoculation. On day 30, COX-2 mRNA level was as low as the detection limit.

Although there were few or no COX-2-immunoreactive cells in the DRG of naive mice (Fig. 5E), there were many immunoreactive cells on day 6 after HSV-1 inoculation (Fig. 5F). COX-2-like immunoreactivities were found around the nuclear membrane (Fig. 5F inset). COX-2-like immunoreactivities were not detected in the DRG on day 30 after inoculation (Fig. 5G). HSV-1 antigen and COX-2 were simultaneously immunostained in single sections to determine cells expressing COX-2 after HSV-1-infection. There were many HSV-1 antigen-positive cells in the DRG on the inoculated side on day 5 after inoculation, and the most of COX-2-like immunoreactivities were in the cells having no HSV-1 antigen (Fig. 5H).

4. Discussion

The present study provides four important findings on the role of PGs in acute herpetic pain and delayed postherpetic pain. First, deficiency in EP_3 receptor, but not EP_1, IP or TP receptors, markedly attenuated acute herpetic pain-related responses, suggesting that EP_3 PGE_2 receptors play an important role in the development and/or maintenance of acute herpetic pain. Preventive administration of the EP_3 receptor antagonist produced a marked inhibition of acute herpetic pain, but the single therapeutic administration was without effects. These results suggest that EP_3 receptors are involved in the development rather than the maintenance of acute herpetic pain. The content of PGE_2, but not 6-keto $PGF_1\alpha$, increased in the infected DRG on days 5–7 after inoculation and the COX inhibitors markedly suppressed the pain-related responses on day 6, suggesting that PGE_2 plays a role in the maintenance of acute herpetic pain. However, since the EP_3 receptor antagonist did not affect the pain-related responses on day 6, it is possible that EP receptor subtype(s) other than EP_3 and EP_1 receptor subtypes are involved in the maintenance of acute herpetic pain. Taken together, these results suggest that the PGE_2 and EP_3 receptor system plays an important role in the

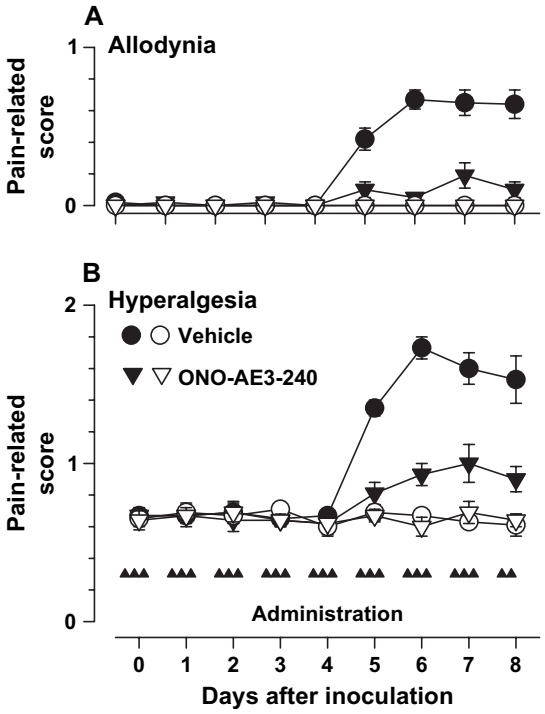

Fig. 3. Effects of repeated administration of ONO-AE3-240 on pain-related responses induced by HSV-1 inoculation. BALB/c mice were inoculated with HSV-1 on the unilateral hind paw and allodynia (A) and hyperalgesia (B) were tested. Closed symbols, inoculated side; open symbols, the contralateral side. Mice were given vehicle ($n=8$) or ONO-AE3-240 ($n=7$) three times (9:00, 15:00 and 21:00 h) daily. Behavioral testing was performed 30–60 min after the second injection of each day (15:30–16:00 h). Each point represents means and SEM. Allodynia and hyperalgesia were significantly different between control and ONO-AE3-240 during a period from day 4 to day 8 post-inoculation (Friedman repeated measures ANOVA on ranks).

development of acute herpetic pain, though other mechanisms may also be involved.

Second, COX-2 appears to play an important role in the induction of acute herpetic pain. Although the expression level of COX-2 mRNA was very low in the DRG of naive mice, HSV-1 infection induced COX-2 mRNA on day 5, a day when HSV-1 actively proliferates in the DRG (Takasaki et al., 2000a,b). The selective COX-2 inhibitors JTE-522 and NS-398 (Wakitani et al., 1998) suppressed pain-related responses on day 6. Diclofenac, a non-selective COX inhibitor (Mitchell et al., 1993), also suppressed pain-related responses, but relatively high doses were needed. Although our results do not provide positive evidence for the involvement of COX-1, the possibility of involvement is not ruled out.

The third important finding is that COX-2-like immunoreactivities were induced in nuclear membrane of many DRG cells, most of which were without HSV antigen. The result suggests that COX-2 is mainly induced in DRG neurons in which HSV-1 does not proliferate. After inoculation, HSV-1 gains access to the peripheral terminals of sensory neurons and moves to sensory ganglia by axonal transport (Cook and Stevens, 1973; Penfold et al., 1994). On day 3 after inoculation, DNA of HSV-1 thymidine kinase is detected in the DRG (Takasaki et al., 2000a), but there are no HSV antigen-positive neurons there (Takasaki et al., 2000b). The level of the viral DNA peaks (Takasaki et al., 2000a) and there are many HSV antigen-positive neurons (present experiment and Takasaki et al., 2000b) on day 5 after inoculation. On day 7 after inoculation, the viral DNA decreases (Takasaki et al., 2000a) and the expression of HSV antigen almost subsides (Takasaki et al., 2000b). With these findings taken into account, the present results suggest that COX-2 expression in the DRG is induced by viral propagation and not by viral invasion. In our preliminary experiments, inflammatory cells were found in the DRG on day 5 after inoculation. Thus, the viral propagation in the DRG results in the infiltration of inflammatory cells, which may release humoral factor(s) to induce COX-2 expression in uninfected neurons. It is not clear why COX-2 was not induced in HSV

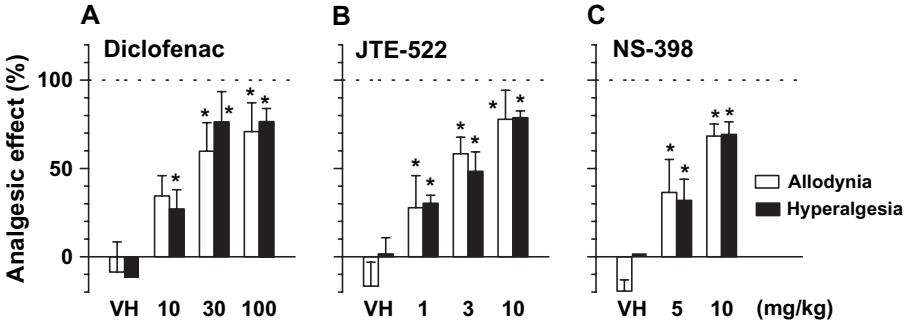

Fig. 4. Effects of COX inhibitors on pain-related responses induced by HSV-1 inoculation. BALB/c mice were inoculated with HSV-1 and the effects of (A) diclofenac sodium (intraperitoneal), (B) JTE-522 (oral), and (C) NS-398 (intraperitoneal) on allodynia (open columns) and hyperalgesia (closed columns) were tested on day 6 after inoculation. See Section 2 for the calculation of analgesic effect. The results show the peak effects. * $P<0.05$ when compared with vehicle (VH) (Dunnett's test after Kruskal–Wallis one-way ANOVA on ranks). The data represents means and SEM ($n=6$ for all groups).

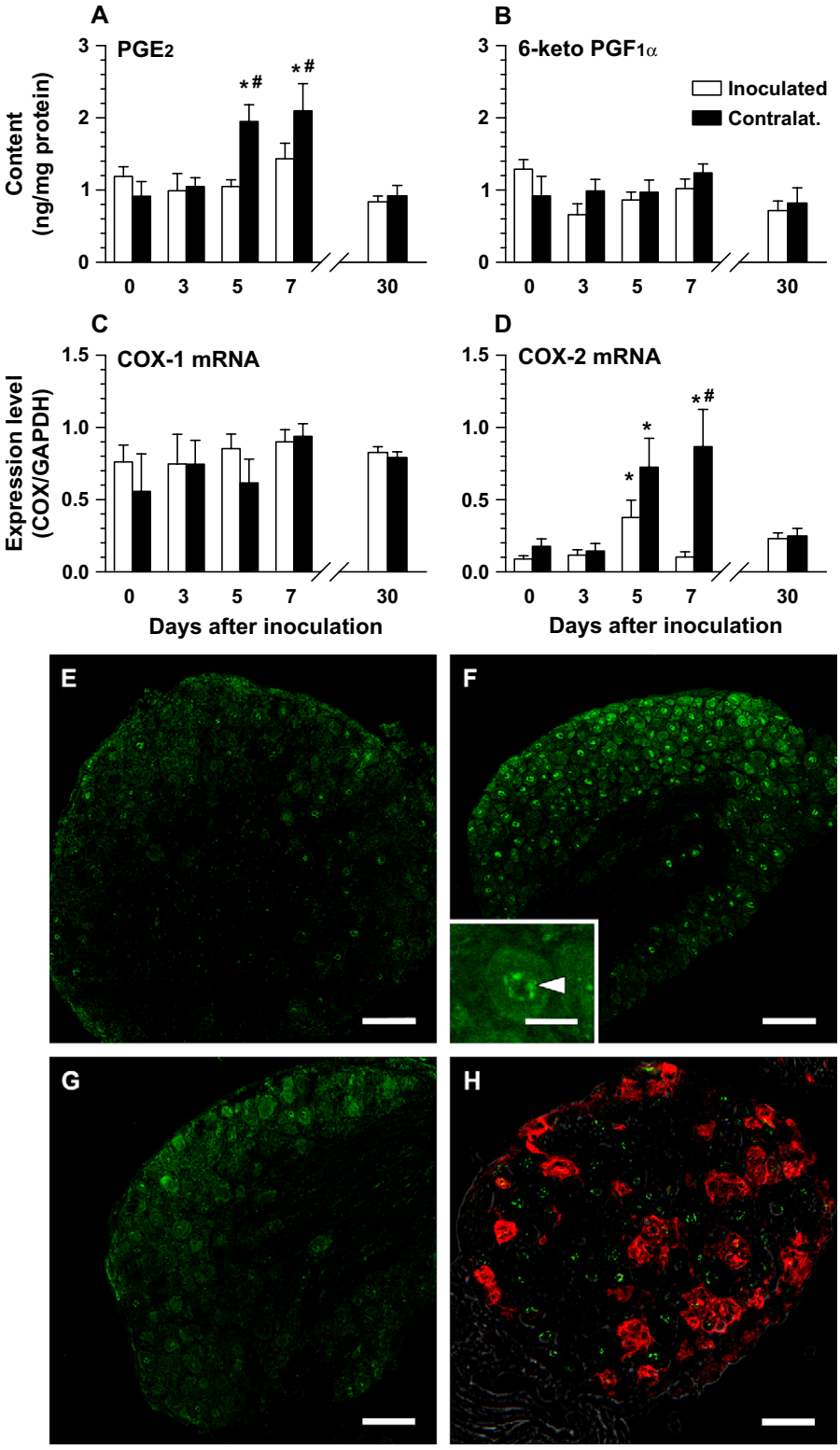

Fig. 5. Effects of HSV-1 inoculation on the levels of prostaglandins, COX mRNAs and COX-2 protein. BALB/c mice were inoculated with HSV-1 on the unilateral hind paw. (A,B) The contents of PGE_2 and 6-keto $PGF_{1\alpha}$ (a stable metabolite of PGI_2) in the DRG were measured by enzyme immunoassay and normalized to total protein. Closed columns, dorsal root ganglia on the inoculated side; open columns, the contralateral side. Each column represents the means and SEM ($n=4$). $^{*}P<0.05$ when compared with day 0 (Dunnett's test). $^{\#}P<0.05$ when compared with contralateral (Dunnett's test). (C,D) The expression levels of COX mRNAs were determined by RT-PCR followed by southern blot hybridization and normalized to the level of GAPDH. (E–G) Immunohistochemical localization of COX-2 in the DRG. E, day 0 (naive). (F) Day 6 post-inoculation, (inset) high magnification images and arrowheads indicate the signal at the nuclear membrane of the DRG cells. (G) Day 30 post-inoculation. (H) Distribution of HSV-1 antigen and COX-2 immunoreactivities in the DRG was determined on day 5 after inoculation. Red (Cy 3) and green (FITC) signals are HSV-1 antigen- and COX-2-like immunoreactivities, respectively (Scale bars: (E)–(H) 100 μm and inset, 25 μm).

antigen-positive neurons. HSV infection decreases membrane excitability and Na current in cultured sensory neurons (Fukuda et al., 1983). HSV-1 inoculation produces hypoalgesia rather than hyperalgesia in rats, in which the virus proliferates in most (about 80%) of DRG neurons (Shiraki et al., 1998). These findings suggest that HSV-1 proliferation decreases the activity and function of infected neurons. It is speculated that HSV antigen-positive neurons cannot induce COX-2 in response to humoral factor(s).

The fourth important finding is that PGs and COX have no important role in the maintenance of the delayed postherpetic pain. Although the contents of PGE_2 and COX-2 expression in the DRG were markedly increased in mice with acute herpetic pain, expression in mice with delayed postherpetic pain was as low as basal level. Diclofenac and the selective COX-2 inhibitor NS-398 at the doses that reduced acute herpetic pain could not ameliorate delayed postherpetic pain. Actually, non-steroidal anti-inflammatory drugs are generally ineffective in postherpetic neuralgia in the clinic. Our present data strongly suggest that COX-2 and PGs are not associated with the maintenance of delayed postherpetic pain.

EP_3 receptor deficiency markedly suppressed pain-related responses without effects on the degree of skin lesions. In addition, there were no essential differences in pain-related responses between BALB/c and C57BL/6J mice, although skin lesions of C57BL/6J mice were significantly slighter than those of BALB/c mice. Therefore, pain-related responses may not be primarily due to rash or cutaneous inflammation. The incidence of postherpetic pain was significantly lower in $EP_3^{-/-}$ mice than in control mice. There are two possible explanations for the effect of EP_3 receptor deficiency. The most likely is that EP_3 receptor deficiency suppresses acute herpetic pain, which results in a decrease in the incidence. This idea is supported by our recent observation that the inhibition of acute herpetic pain by repeated medication of analgesics decreases the incidence of postherpetic pain in mice (Kuraishi et al., 2004). Another explanation is that EP_3 receptor itself is involved in the development of postherpetic pain. Partial sciatic nerve ligation induces long-lasting mechanical allodynia in mice, which is suppressed by EP_3 receptor deficiency and repeated administration of COX inhibitors immediately after operation (Sasamura et al., 1999; our unpublished observation). However, since EP_3 receptor deficiency did not abolish the postherpetic pain, EP_3 receptor may not play a key role in the development of postherpetic pain. Repeated administration of diclofenac to inhibit acute herpetic pain is lethal to mice probably because of gastrointestinal affection (Kuraishi et al., 2004). Thus, EP_3 receptor antagonist may be better than conventional COX inhibitors for the treatment of acute herpetic pain.

EP_3 receptors are ubiquitously expressed in the central and peripheral nervous systems. Half or more neurons in the DRG express the mRNA and protein of EP_3 receptor in rodents (Sugimoto et al., 1994; Nakamura et al., 2000). Therefore, it is possible that PGE_2 acts on the EP_3 receptors in the DRG neurons to produce a pain state. It will be interesting to determine whether PGE_2 has autocrine and/or paracrine actions in the DRG. A whole-cell patch clamp study revealed that application of PGE_2 and EP_3 receptor agonist depolarized the membrane of the large dorsal raphe neurons (Momiyama et al., 1996), suggesting that activation of EP_3 receptor leads to excitation of dorsal raphe neurons. PGE_2 also increases the excitability of neonatal rat dorsal root ganglion neurons via cAMP-protein kinase A cascade (England et al., 1996). Considering these findings and EP_3 splicing variant (Namba et al., 1993; Kotani et al., 1995), involvement of EP_3 receptor coupled to the elevation in intracellular cAMP levels would be suggested.

The incidence of delayed postherpetic pain was higher in C57BL/6J mice than in BALB/c mice. Although we cannot deny the influence of acyclovir treatment on the different incidence of the two strains of mice, the result raises the possibility of the involvement of genetic and/or genomic factors on the induction of delayed postherpetic pain. Skin lesions were milder in C56BL/6J mice than in BALB/c mice, in which the difference in T cell repertory may be involved. Human major histocompatibility complex haplotype has been shown to be associated with the incidence of postherpetic neuralgia (Sato-Takeda et al., 2004). We are now studying the association of major histocompatibility complex with the incidence of delayed postherpetic pain.

In summary, our results show that PGE_2 and EP_3 receptors are responsible for the development but not maintenance of acute herpetic pain. COX-2 induced in uninfected neurons in the DRG may be involved in the production of PGE_2. COX-2 and EP_3 receptor may be preferable targets for treatment of acute herpetic pain and prevention of postherpetic neuralgia.

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