Algogenic Effects of the Extract of the Tumor Mass Isolated from Mice with Orthotopic Melanoma Inoculation

HongWei Zhang¹, Takashi Sasamura¹, Yuko Iida¹, Hiroshi Nojima¹,
Jun Murata², Ikuo Saiki² and Yasushi Kuraishi¹

¹*Department of Applied Pharmacology, Faculty of Pharmaceutical Sciences,*

²*Department of Pathogenic Biochemistry, Institute of Natural Medicine,
Toyama Medical and Pharmaceutical University, Toyama, Japan*

(Received 27 April 2001)

Abstract

The present study was conducted to examine whether an injection of the extract from inoculated melanoma in mice would elicit painful responses in naive mice. Furthermore, the responses were compared with the inflammatory responses induced by carrageenan. Sixteen to twenty days after intraplantar inoculation of melanoma cells into the hind paw of mice, the tumor mass was isolated and the extract was prepared with homogenization in water. An intraplantar injection of the extract into the hind paw of naive mice elicited the following responses in the injected paw: 1) licking behavior, which peaked at 5–15 min after injection and then subsided within 30 min, 2) mechanical hyperalgesia, which peaked at 15 min after injection and then subsided within 5 hr, and 3) paw edema, which peaked at 45 min after injection and then completely recovered within 6 h. Carrageenan-induced mechanical hyperalgesia and paw edema were suppressed by diclofenac, a cyclooxygenase inhibitor, at doses of 10 and 30 mg/kg. Diclofenac at the same doses partly suppressed the paw edema and licking behavior, but not the mechanical hyperalgesia, induced by melanoma extract. These results suggest that more than one factor mediate the algogenic effects of melanoma extract and probably some substances other than prostaglandins released from melanoma mass evoke the mechanical hyperalgesia.

Key words: cancer pain, melanoma, hyperalgesia, licking behavior, carrageenan, mice

INTRODUCTION

Pain is one of the primary complaints among cancer patients. Approximately 70% of cancer patients suffer from pain and up to 90% must cope with chronic pain syndromes in the terminal stages of the

disease^{1,2}). However, unrelieved pain still remains a great problem. The general lack of understanding of the mechanisms involved in the occurrence of cancer pain prevents the development of new treatments for these patients. All pain is not necessarily elicited in the same way; different pain possesses distinct neurochemical changes^{3–8}). Above all, cancer pain increases

progressively and intensively with time. Thus, some inducible intrinsic substances may be responsible for the severe pain at the progressive stage of cancer. Although various useful animal models for the study of neuropathic, inflammatory and herpetic pain have been developed^{9–13}, the authorized animal model for the study of cancer pain has been scarcely reported¹⁴. Recently, we have developed a cancer pain model by the orthotopic inoculation of B16-BL6 melanoma cells into the plantar region of the hind paw of C57BL/6 mice¹⁵. Mice inoculated with melanoma exhibit thermal hyperalgesia and licking behavior following the tumor growth. The hyperalgesia is developed as biphasic responses of the early phase (light pain phase during 7–14 days after inoculation) and the late phase (severe pain phase during 15–21 days). In the present study, we examined whether an injection of the extract from inoculated melanoma in mice would elicit painful responses in naive mice. Furthermore, to clarify the involvement of inflammation in the development of pain responses induced by the extract from tumor mass, we compared the effects of a non-steroidal antiinflammatory drug on the melanoma extract-induced pain responses with those on the inflammatory pain responses induced by carrageenan.

MATERIALS AND METHODS

Animals: Male C57BL/6 mice (Japan SLC, Inc., Shizuoka, Japan) were used. They were 6 weeks old at the start of experiments. Mice were housed in a room under controlled temperature ($22 \pm 2^\circ\text{C}$), humidity ($55 \pm 10\%$) and light (light on 07:00–19:00 h). Food and water were freely available. The experimental procedures for mice were conducted in accordance with the guidelines of the Guiding Principles for the Care and Use of Laboratory Animals approved by the Committee for Animal Experiments in Toyama Medical and Pharmaceutical University.

Preparation of melanoma extract: B16-BL6 melanoma cells were derived from C57BL/6 mouse (B16-BL6 melanoma cell line was kindly provided by Dr. I.J. Fidler, Anderson Cancer Center, Houston, Tex, USA). They were inoculated by an intraplantar injection of Eagle's minimum essential medium ($20 \mu\text{l}$) containing 2×10^5 cells into the left hind paw of

mice. Sixteen to twenty days after injection, mice were killed under anesthesia, and then the melanoma mass was isolated. After homogenization in purified water and subsequent centrifugation at $17,500 \times g$ for 30 min at 4°C , the supernatant was transferred into plastic tubes for lyophilization. The lyophilized melanoma extract was stored at -30°C , and on the day of experiment it was dissolved in sterile saline at a concentration of 50 mg or 100 mg extract per ml and kept on ice until injected.

Evaluation of mechanical hyperalgesia: The evaluation of mechanical hyperalgesia of the hind paw was carried out essentially according to the method of Takasaki et al.¹⁶. Each mouse was placed individually in a plastic cage ($10 \times 8 \times 18 \text{ cm}$) with a mesh metal floor. The experimenter could reach the plantar surface of the paw from beneath without being noticed by the mouse. After acclimation for at least 15 min, a von Frey filament with bending force of 1.2 g (noxious stimulation) was pressed perpendicularly against the plantar skin and held for 2–3 s with it slightly buckled. The stimulation of the same intensity was applied five times to each hind paw at intervals of several seconds. The responses to these stimuli were ranked as follows: 0, no response; 1, move away from von Frey filament; and 2, immediate flinching or licking of the hind paw. Nociceptive score was calculated as described below:

$$\text{Nociceptive score (\%)} = \frac{\Sigma (\text{average score of each animal})}{2 \times (\text{numbers of animals tested})} \times 100$$

Prior to the experiment on the nociception induced by either carrageenan or melanoma extract, mice were habituated to the test procedure for 3 or 4 days.

Observation of licking behavior: Each mouse was placed in a acrylic cage ($26 \times 18 \times 30 \text{ cm}$) composed of 4 cells ($13 \times 9 \times 30 \text{ cm}$) for 1 h for acclimation. Immediately after intraplantar injection, they were put back to the same cell. Their behaviors were videotaped for 1 h and observed later by playing back the tape. During the videotaping period, any experimenter was kept out from the observation room. The licking duration in 5-min intervals and the total licking time per 1 h were measured.

Measurement of paw volume: The displacement volume (ml) of the glabrous region of each hind paw was assessed using a plethysmometer. Briefly, the paw was put into a liquid reservoir up to the level of the heel and the displacement volume was read.

The animals were used for only one measurement in each experiment. All experimental procedures were conducted in accordance with the ethical guidelines of the Ethical Committee of the International Association for the Study of Pain¹⁷.

Reagents: Agents used were γ -carrageenan (Wako Pure Chemical Industries, Ltd., Japan, prepared as an injectable suspension of 4%), diclofenac sodium (Research Biochemical International, Natick, MA, USA, prepared as 1 mg/ml and 3 mg/ml saline solution). Melanoma extract and 4% carrageenan suspension were injected intraplantarly at a volume of 20 μ l into the unilateral hind paw. Diclofenac was administered intraperitoneally at doses of 10 and 30 mg/kg in a volume of 10 ml/kg body weight. All saline controls received equivolume of physiological saline. At various time points after administration of the melanoma extract, mechanical hyperalgesia, paw edema and licking behavior were measured. After intraplantar carrageenan, the mechanical hyperalgesia and paw edema were measured.

Statistical analysis: Data were presented as mean $\pm$ S.E.M. Results were analyzed with Dunnett's multiple comparisons after one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA (RM-ANOVA): $p < 0.05$ was considered significant.

RESULTS

Licking behavior, mechanical hyperalgesia and paw edema elicited by melanoma extract

When given by intraplantar injection at concentrations of 1 mg and 2 mg per 20 μ l to naive mice, the melanoma extract evoked licking response, which peaked at 10–15 min and 5–10 min, respectively, and almost disappeared within 30 min after injection (Fig. 1a). The extract also dramatically elicited mechanical hyperalgesia in a dose-dependent manner (Fig. 1b). Following the injection, mechanical hyperalgesia was immediately developed and reached a

Fig. 1 Time-course of licking behavior (a), mechanical hyperalgesia (b), and paw edema (c) induced by an intraplantar injection of melanoma extract in naive mice. The extract of 1 mg (●) or 2 mg (▲) was injected subcutaneously. An intraplantar injection of saline (○) served as the control group. Values represent the means $\pm$ S.E.M. of 5–6 animals.

maximal level at 15 min; nociceptive score increased from $20 \pm 4\%$ (prior to injection; mean $\pm$ S.E.M., $n = 6$) to $58 \pm 5\%$ and $78 \pm 5\%$ (15 min after extract of 1 and 2 mg, respectively), and then sharply decreased within 75 min. Since 1.5 h after injection, the hyperalgesia gradually decreased and completely subsided by 5 h. Simultaneously, the melanoma extract elicited paw edema with a maximal volume at 45 min after injection; the paw volume increased from 0.145 ± 0.001 ml (prior to injection; mean $\pm$ S.E.M., $n = 6$) to 0.20 ± 0.003 ml and 0.24 ± 0.004 ml (45 min after extract of 1 and 2 mg, respectively) and then decreased to the basal level by around 6 h (Fig. 1c). On the other hand, neither licking response, hyper-

Fig. 2 Effects of diclofenac on the mechanical hyperalgesia (a, b) and paw edema (c, d) induced by an intraplantar injection of 4% carrageenan (a, c) or 2 mg melanoma extract (b, d). Diclofenac at doses of 10 (●), 30 (▲) mg/kg or saline (○) was intraperitoneally injected 1 h prior to intraplantar melanoma extract or 3 h after carrageenan challenge (indicated by an arrow). Values represent the mean $\pm$ S.E.M. ($n = 5-6$). Statistical analysis by two-way RM-ANOVA revealed significant overall inhibitory effect of diclofenac on melanoma extract-induced paw edema ($F_{2,14} = 9.86$, $p < 0.01$), carrageenan-induced hyperalgesia ($F_{2,12} = 14.74$, $p < 0.001$) and carrageenan-induced paw edema ($F_{2,13} = 7.14$, $p < 0.01$) as compared with each saline group, but melanoma extract-induced mechanical hyperalgesia was not affected by diclofenac at the given doses.

algnesia nor paw edema was elicited by an intraplantar injection of saline into the hind paw of naive mice (Fig. 1).

Effects of diclofenac on licking behavior, mechanical hyperalgesia and paw edema induced by melanoma extract and carrageenan

In order to clarify the involvement of prostaglandins in the development of the melanoma extract-induced licking behavior, hyperalgesia and paw edema, we examined the effects of the cyclooxygenase inhibitor diclofenac. These diclofenac effects were compared with those on mechanical hyperalgesia and paw edema after carrageenan. In carrageenan-injected mice, the licking of the injected paw was not observed throughout the experimental period. When adminis-

Fig. 3 Effect of diclofenac on the licking behavior induced by melanoma extract in mice. Diclofenac (DIC) at doses of 10 (●), 30 (▲) mg/kg or saline (○) was intraperitoneally injected 1 h prior to an intraplantar injection of 2 mg melanoma extract. Time course of licking behavior per 5-min (a) and total licking duration for 1 h (b) were shown. * $p < 0.05$ when compared with saline (Dunnett's test). Values represent the means $\pm$ S.E.M. of 4-9 animals.

tered 3 h after intraplantar carrageenan, diclofenac (10 and 30 mg/kg) dose-dependently attenuated mechanical hyperalgesia and paw edema induced by carrageenan (Fig. 2a, c). On the other hand, an administration of the same doses of diclofenac 1 h prior to intraplantar melanoma extract had no significant effect on the mechanical hyperalgesia (Fig. 2b). It slightly inhibited the paw edema at a dose of 30 mg/kg, but not 10 mg/kg (Fig. 2d). Diclofenac also significantly suppressed melanoma extract-induced licking behavior at a dose of 30 mg/kg, but not 10 mg/kg (Fig. 3).

DISCUSSION

This experiment was conducted to clarify whether some factors released from the tumor mass could induce painful behaviors. Thus, the nociceptive responses induced by the extract of the melanoma mass, which developed in the intraplantar region of the hind paw of mice, were examined. In the present study, we demonstrated that an intraplantar injection of the extract elicited licking behavior, mechanical hyperalgesia and edema in the injected paw. The time-course of the development of the hyperalgesia was largely similar to that of the edema, which peaked within 45 min after injection and continued for several hours. However, the licking behavior

appeared in a short-time manner. These results raised the possibility that the mechanical hyperalgesia induced by the injection of melanoma extract resulted from the peripheral inflammation. Therefore, we examined the effects of a cyclooxygenase inhibitor diclofenac on the nociceptive responses and edema induced by melanoma extract and carrageenan, because prostaglandins play an important role in promoting the signs and symptoms of inflammation and in enhancing the responsiveness of C-fibers to algesic stimuli^{18,19}).

Carrageenan-induced inflammation is widely used in animal experiments¹¹), and prostaglandins are generally accepted to be involved in the mechanism^{20,21}). Thus, in order to confirm the contribution of prostaglandins to the painful responses induced by the melanoma extract, a comparative study between the inflammatory responses induced by melanoma extract and carrageenan was performed.

First, we found that the nociceptive responses and paw edema induced by melanoma extract were different from those by carrageenan in onset, magnitude and duration. Intraplantar injection of melanoma extract evoked the mechanical hyperalgesia and paw edema, which peaked at 15 min and 45 min, respectively, and then recovered to pre-injection values within 5–6 hours. On the other hand, the peak times for carrageenan-induced mechanical hyperalgesia and paw edema were around 6 h after injection. Although the maximal value of the paw edema by carrageenan was almost the same as that by melanoma extract, the maximal score of the carrageenan-induced hyperalgesia was only 60% of that induced by 2 mg melanoma extract. Moreover, carrageenan-induced nociceptive responses did not recover within several days (data not shown).

Second, we found that the inhibitory effects of diclofenac^{22,23}) were different between melanoma extract- and carrageenan-induced hyperalgesia. Although diclofenac significantly suppressed the paw edema induced by melanoma extract and carrageenan and the licking behavior evoked by melanoma extract, the effects on the mechanical hyperalgesia were quite different. When injected 3 h after carrageenan, diclofenac caused a considerable reduction of the

mechanical hyperalgesia in the inflamed paw, and almost recovered it to normal level within 2 h at a dose of 30 mg/kg. In contrast, the development of the melanoma extract-induced mechanical hyperalgesia was not significantly affected by pretreatment with diclofenac, although the hyperalgesia tended to be attenuated. These results suggest that the mechanisms of hyperalgesia induced by the melanoma extract are different from those of carrageenan. At least, prostaglandins may not be primarily responsible for the melanoma extract-induced hyperalgesia.

To some extent, the characteristics of melanoma extract-induced nociception seem to be similar to those elicited by capsaicin. Topical application or intradermal injection of capsaicin produce intense pain for several minutes and lead to a state of hyperalgesia that last for several hours^{24–26}). It has been demonstrated that the pain sensation and the development of hyperalgesia evoked by capsaicin depend on the activation of afferent C-nociceptors^{25,27}). In this context, some capsaicin-like substances released from the melanoma mass might be involved in the mechanism of the occurrence of painful behaviors.

Honore, et al.³) demonstrated that cancer induced a unique persistent pain state that was quite different from that of the inflammatory and neuropathic pain. In the inflammatory pain model, there was a significant up-regulation of spinal substance P and calcitonin gene-related peptide in the dorsal horn. In neuropathic pain model, galanin and neuropeptide Y were dramatically up-regulated in dorsal root ganglionic neurons. On the other hand, none of the above changes was found in cancer pain, the characteristic neurochemical change of cancer pain was the upregulation of glial fibrillary acidic protein in the spinal cord. Therefore, the cancer-induced pain is a distinct pain entity. In the present study, we demonstrated that the extract from the melanoma mass evoked painful responses and some inducible substances other than prostaglandins may be involved in the mechanism of the cancer-induced pain. The identification of the algesic substances contained in the melanoma extract remains to be carried out.

In conclusion, the extract of the melanoma mass isolated from the inoculated melanoma evoked

painful responses in naive mice. Some substances released from melanoma mass may be involved in the development of cancer pain with a manner different from that of the inflammatory pain.

References

- 1) Shvartzman, P., Pharmacological treatment of cancer pain, *Israel Med. Assoc. J.*, 2 (2000) 536-539.
- 2) Portenoy, R.K. and Lesage, P., Management of cancer pain, *Lancet*, 353 (1999) 1695-1700.
- 3) Honore, P., Rogers, S.D., Schwei, M.J., Salk-Johnson, J.L., Luger, N.M., Sabino, M.C., Clohisey, D.R. and Mantyh, P.W., Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons, *Neuroscience*, 98 (2000) 585-598.
- 4) Donnerer, J., Schuligoi, R. and Stein, C., Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidences for a regulatory function of nerve growth factor in vivo, *Neuroscience*, 49 (1992) 693-698.
- 5) Galeazza, M.T., Garry, M.G., Yost, H.J., Strait, K.A., Hargreaves, K.M. and Seybold, V.S., Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation, *Neuroscience*, 66 (1995) 443-458.
- 6) Hokfelt, T., Zhang, X. and Wiesenfeld-Hallin, Z., Messenger plasticity in primary sensory neurons following axotomy and its functional implications, *Trends Neurosci.*, 17 (1994) 22-30.
- 7) Wakisaka, S., Kajander, K.C. and Bennett, G.J., Effects of peripheral nerve injuries and tissue inflammation on the levels of neuropeptide Y-like immunoreactivity in rat primary afferent neurons, *Brain Res.*, 598 (1992) 349-352.
- 8) Zhang, X., Bean, A.J., Wiesenfeld-Hallin, Z., Xu, X.-J. and Hokfelt T., Ultrastructural studies on peptides in the dorsal horn of the rat spinal cord-III. Effects of peripheral axotomy with special reference to galanin, *Neuroscience* 64 (1995) 893-915.
- 9) Mosconi, T. and Kruger, L., Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations, *Pain*, 64 (1996) 37-57.
- 10) Seltzer, Z., Dubner, R. and Shir, Y., A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury, *Pain*, 43 (1990) 205-218.
- 11) Levy, L., Carrageenan paw edema in the mouse, *Life Sci.*, 8 (1969) 601-606.
- 12) Roubenoff, R., Freeman, L.M., Smith, D.E., Abad, L.W., Dinarello, C.A. and Kehayias, J.J., Adjuvant arthritis as a model of inflammatory cachexia, *Arthritis Rheumat.*, 40 (1997) 534-539.
- 13) Dubuisson, D. and Dennis, S.G., The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats, *Pain*, 4 (1977) 161-174.
- 14) Mercadante, S., Malignant bone pain: pathophysiology and treatment, *Pain*, 69 (1997) 1-18.
- 15) Nojima, H., Sasamura, T., Fujii, H., Murata, J., Saiki, I., Tohda, C., Narumiya, S. and Kuraishi, Y., IP prostaglandin receptor involvement in thermal hyperalgesia in neuropathic- and cancer-pain model mice, *Soc. Neurosci., Abstr.*, 26 (2000) 1961.
- 16) Takasaki, I., Andoh, T., Shiraki, K. and Kuraishi, Y., Allodynia and hyperalgesia induced by herpes simplex virus type-1 infection in mice, *Pain*, 86 (2000) 95-101.
- 17) Zimmermann, M., Ethical guidelines for investigations of experimental pain in conscious animals, *Pain*, 16 (1983) 109-110.
- 18) Martin, H.A., Basbaum, A.I., Kwiat, G.C., Goetzl, E.J. and Levine, J.D., Leukotriene and prostaglandin sensitization of cutaneous high-threshold C- and A-delta mechanonociceptors in the hairy skin of rat hindlimbs, *Neuroscience*, 22 (1987) 651-659.
- 19) Cohen, R.H. and Perl, E.R., Contributions of arachidonic acid derivatives and substance P to the sensitization of cutaneous nociceptors, *J. Neurophysiol.*, 64 (1990) 457-464.
- 20) Nantel, F., Denis, D., Gordon, R., Northey, A., Cirino, M., Metters, K.M. and Chan, C.C., Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation, *Br. J. Pharmacol.*, 128 (1999) 853-859.
- 21) Oh-ishi, S., Analysis of chemical mediators involved in acute inflammatory reaction with the rat pleurisy model, *Folia Pharmacol. Japon.*, 110 (1997) 59-68.
- 22) Menasse, R., Hedwall, P.R., Kraetz, J., Pericin, C., Riesterer, L., Sallmann A., Ziel, R. and Jaques, R., Pharmacological properties of diclofenac sodium and its metabolites, *Scand. J. Rheumatol., Suppl.*, 22 (1978) 5-16.
- 23) Attal, N., Kayser, V., Eschaliere, A., Benoist, J.M. and Guilbaud, G., Behavioral and electrophysiological evidence for an analgesic effect of a non-steroidal anti-inflammatory agent, sodium diclofenac, *Pain*, 35 (1988) 341-348.

- 24) Lamotte, R.H., Shain, C.N., Simone, D.A. and Tsai, E.P., Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms, *J. Neurophysiol.*, 66 (1991) 190-211.
- 25) Torebjork, H.E., Lundberg, L.E.R. and Lamotte, R.H., Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans, *J. Physiol.*, 448 (1992) 765-780.
- 26) Schmelz, M. and Kress, M., Topical acetylsalicylate attenuates capsaicin induced pain, flare and allodynia but not thermal hyperalgesia, *Neurosci. Lett.*, 214 (1996) 72-74.
- 27) Koltzenburg, M., Lundberg, L.E.R. and Torebjork, H.E., Dynamic and static components of mechanical hyperalgesia in human hairy skin, *Pain*, 51 (1992) 207-219.

Address for correspondence : Dr. Yasushi Kuraishi
 Department of Applied Pharmacology
 Faculty of Pharmaceutical Sciences
 Toyama Medical and Pharmaceutical University
 2630 Sugitani, Toyama 930-0194, Japan
 Tel: +81-76-434-7510 Fax: +81-76-434-5045
 e-mail: kuraisiy@ms.toyama-mpu.ac.jp