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Morphine analgesia suppresses tumor growth and metastasis in a mouse model of cancer pain produced by orthotopic tumor inoculation

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Abstract

The present study was conducted to clarify whether relief from cancer pain by morphine would suppress tumor growth and metastasis. When given orthotopic inoculation of B16–BL6 melanoma cells into the hind paw, C57BL/6 mice showed moderate and marked hyperalgesia on days 7–10 and from day 14 post-inoculation, respectively. The volume of inoculated hind paw was increased exponentially as a function of time from day 8 post-inoculation, a phenomena being due to melanoma growth. Lung metastasis was apparent after day 12 post-inoculation. On day 16 post-inoculation, the hyperalgesia was completely inhibited by subcutaneous injection of morphine hydrochloride (5 and 10 mg/kg). The tumor growth and lung metastasis were markedly inhibited by repeated administration of morphine (5 and 10 mg/kg daily for 6 days) and also by the neurectomy of sciatic nerve innervating the inoculated region. The results suggest that relief from cancer pain by morphine inhibits tumor growth and metastasis. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Morphine; Cancer pain; Hyperalgesia; Tumor growth; Tumor metastasis; (Mouse)

1. Introduction

One third of patients with metastatic cancer complain of pain and 60–90% of patients with advanced cancer have substantial pain (Forey, 1985; Levy, 1996). Morphine is used to relieve pains of patients with terminal cancer, mainly to improve quality of life. Morphine effectively relieves cancer pain (Radbruch et al., 1996), although the high doses induce adverse effects, such as sedation, nausea, constipation and urinary retention (Levy, 1996; Bruera and Lawlor, 1997). Conveniently, morphine inhibits the growth of tumor cells in vitro (Ishikawa et al., 1993; Sueoka et al., 1996). However, morphine enhances tumor growth and reduces survival of tumor-bearing rats (Lewis et al., 1984), which may be partly due to the suppression of immune systems (Ishikawa et al., 1993; Fecho et al., 1996; Gavériaux-Ruff et al., 1998). The promotion of Fas-mediated apoptosis of immune cells may be involved in the immunosuppression by morphine (Yin et al., 1999). On the other hand, painful cancer is possible to aggravate cancer because painful stress also reduces survival of tumor-bearing rats (Lewis et al., 1983). In addition, stress increases the incidence of carcinogenesis, which is inhibited by opioid antagonist (Tejwani et al., 1991). These findings raise the possibility that immoderate use of morphine aggravates cancer. Therefore, it should be determined whether analgesic doses of morphine would affect the growth and metastasis of tumor cells in animals with cancer pain.

At present, a few murine models of cancer pain have been reported. Schwei et al. (1999) proposed a murine model of bone cancer pain produced by intramedullary injection of osteolytic sarcoma cells into the femur. Recently, a new model of cancer pain produced by implantation of fibrosarcoma cells into the mouse calcaneus bone has been described (Wacnik et al., 2001; Cain et al., 2001). The latter model provides an approach for quantifying the behavioral, biochemical and electrophysiological consequences of tumor–nerve interactions. However, they are extremely difficult to evaluate the metastasis of tumor cells. Mice inoculated a highly invasive B16–BL6 melanoma

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have been frequently utilized as a spontaneous lung metastasis model (Saiki, 1997). Here, we report that the mice given orthotopic inoculation of the melanoma cells show pain symptoms and that analgesic doses of morphine inhibit the growth and metastasis of tumor cells.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (6 weeks old at the inoculation of melanoma; Japan SLC, Shizuoka, Japan) were used. They were kept under controlled temperature (22 ± 1 °C), humidity (55 ± 10%) and light (light on from 0700 to 1900 h). Food (CE-2, Japan Clea, Tokyo, Japan) and water were freely available. The study was approved by The Animal Care and Use committee at Toyama Medical and Pharmaceutical University and performed in accordance with the guidelines for the Care and Use of Laboratory Animals.

2.2. Tumor inoculation

B16–BL6 cells, a highly invasive variant of B16 melanoma derived from C57BL/6 mouse (Poste et al., 1980), were kindly provided by Dr. I.J. Fidler, MD, Anderson Cancer Center, Houston, TX, USA. B16–BL6 melanoma cells were incubated as monolayer cultures in Eagle's minimum essential medium containing 5% fetal bovine serum. The melanoma (2×10^5 cells) suspended in phosphate-buffered saline was injected subcutaneously into the plantar region of the unilateral hind paw of the mouse in a volume of 20 μl and phosphate-buffered saline into the contralateral hind paw. To assess the growth of melanoma in situ, the volume of glabrous region of the hind paw was plethysmographically determined.

2.3. Nociceptive tests

Nociceptive tests were performed according to the guidelines published in a Guest Editorial in Pain on ethical standards for investigations of experimental pain in animals (Zimmermann, 1983). For the test of thermal hyperalgesia, radiant heat was applied to the plantar region of the mouse hind paw and the latency of paw-withdrawal response was determined, using a tail-flick apparatus (Ugo Basile, Milan, Italy). The intensity of radiant heat was adjusted to elicit the response around 13 s in normal mice. The average of three trials was regarded as nociceptive latency.

For assessing of spontaneous pain, the behaviors of mice were videotaped for 1 h with any experimenter kept out from the observation room. The recording was done after 1-h acclimation from 1600 to 1900 h. The tapes were played back to count the frequency of licking of the hind paw. The effect of morphine was examined on day 22 post-inoculation. After 1-h acclimation, the control recording was done for 1 h. The mice were given subcutaneously morphine and then recorded from 1 to 2 h after.

2.4. Metastasis assay

Pulmonary metastasis of B16–BL6 cells inoculated into the hind paw was assayed as described (Saiki et al., 1989). In brief, primary tumors were surgically removed under anesthesia on day 25 post-inoculation, except one experiment in which tumors were excised on days 4, 8, 12, 16, 20 and 24 post-inoculation. Two weeks after the excision, the mice were killed and the lungs were removed. After fixed in Bouin's solution, the tumor nodules in the lung were counted under a stereoscopic microscope.

2.5. Neurectomy of sciatic nerve

On day 15 post-inoculation, the sciatic nerve innervating the inoculated region was exposed at the middle of thigh under ether anesthesia and a length of 2–3 mm of the nerve was cut and removed. In mice of sham-operated control, the sciatic nerve was exposed without excision.

2.6. In vitro cell growth assay

B16–BL6 melanoma cells (500, 1000 or 2000 cells/well) were seeded in 48-well plate containing Eagle's minimum essential medium and 5% fetal bovine serum. After morphine was added to the well, the plates were incubated at 37 °C for 48 h. The number of cells was determined using WST-1 cell counting kit (Wako, Osaka, Japan), as described (Murata et al., 1997).

2.7. Drugs

For systemic administration, morphine hydrochloride (Sankyo, Tokyo, Japan) and diclofenac sodium (Research Biochemical International, Natick, USA) were dissolved in physiological saline (Otsuka, Tokushima, Japan) just before use. Dosages are given in terms of the weights of their respective salts. In in vitro experiments, morphine was dissolved in the incubation medium. λ-Carrageenan (Wako) was suspended in saline and its 4% solution was injected subcutaneously into the plantar region of the hind paw in a volume of 20 μl.

2.8. Statistical analysis

Data were presented as the mean ± S.E.M., except for in vitro experiments in which S.D. was given together with mean. Results were analyzed with paired *t*-test, Student's *t*-test or Dunnett's multiple comparisons after one-way analysis of variance (ANOVA) or two-way repeated measures analysis of variance (RM-ANOVA): $P < 0.05$ was considered significant.

3. Results

3.1. Nociceptive responses after melanoma inoculation

When B16–BL6 melanoma was inoculated into the plantar region of the hind paw, there were no apparent changes in size of melanoma tissue until day 7 post-inoculation. However, melanoma became apparent as a black nodule around day 8 post-inoculation (Fig. 1A), the volume of the inoculated paw being 109.5 ± 3.1% (n = 8) of pre-inoculation on day 8 post-inoculation. Thereafter, the paw volume was increased exponentially as a function of time (Fig. 1A,B); on day 16 post-inoculation, the volume of the inoculated paw was 175.9 ± 11.0% (n = 8) of pre-inoculation.

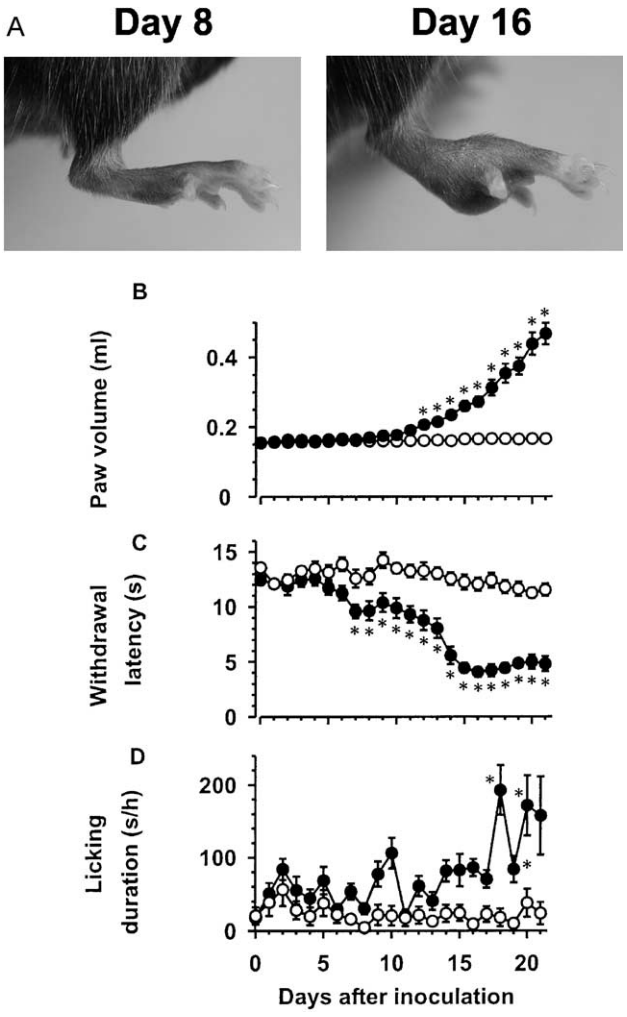

Fig. 1. Tumor growth and nociceptive behaviors after orthotopic inoculation with melanoma. B16–BL6 cells (2×10^5 cells/site) were inoculated subcutaneously into the plantar region of the unilateral hind paw. (A) Lateral aspect of the hind paw on 8 and 16 days after inoculation. (B) Increase in the volume of the inoculated hind paw, indicating the tumor growth. The volume of the glabrous region of the hind paw was plethysmographically measured. (C) The development of thermal hyperalgesia in the inoculated hind paw. Radiant heat stimulation was applied to the plantar region of the hind paw and the latency of withdrawal response was determined. (D) Increase of licking duration of the inoculated hind paw. Licking of the hind paws was measured for 1 h every day. Closed circles, the inoculated hind paw; open circles, the contralateral hind paw. Each point represents the mean and S.E.M. of 7–8 animals. * $P < 0.05$ as compared with the contralateral hind paw (paired t-test).

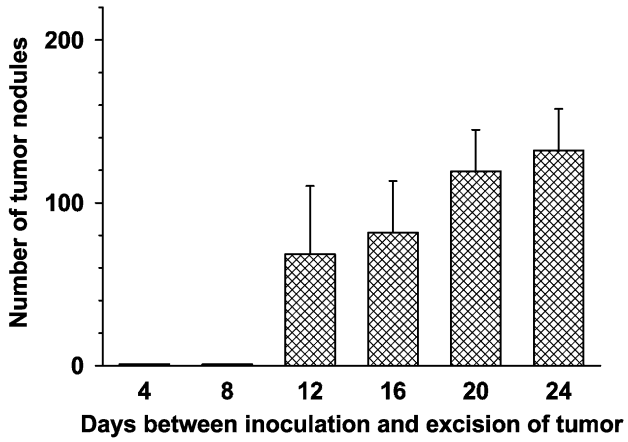

Fig. 2. Lung metastasis after melanoma inoculation into the hind paw. The mice were inoculated with melanoma cells, as described in Fig. 1. Primary tumors were removed 4–24 days after the inoculation, and 2 weeks later, the number of tumor nodules in the lung was counted. Each value represents the mean and S.E.M. of 8–13 animals.

For the test of thermal hyperalgesia, we examined the withdrawal response of the mouse to radiant heat stimulation. The latency of withdrawal response of the inoculated hind paw to heat stimulation was moderately shortened on days 7–10 post-inoculation (early phase) and markedly shortened from day 14 to at least day 21 post-inoculation (late phase) (Fig. 1C). The withdrawal latency on day 8 and day 16 post-inoculation was 77.3 ± 6.4 and 32.6 ± 3.4% (n = 8) of the pre-inoculation latency, respectively. To compare tumor inoculation with acute inflammation, carrageenan was injected into the hind paw to induce inflammatory hyperalgesia; the withdrawal latency and paw volume were 80.9 ± 4.3% and 156.0 ± 6.0% (n = 13), respectively, of pre-injection at 5 h after carrageenan injection.

There were no apparent differences in the incidence of licking between the inoculated and contralateral hind paws until day 8 post-inoculation. Although the frequency of licking of the inoculated hind paw varied from day to day, it was relatively higher on the inoculated side than on the contralateral side from day 9 to day 17 post-inoculation and significantly higher on the inoculated side from day 18 post-inoculation (Fig. 1D).

To determine when inoculated melanoma cells begin to metastasize, primary tumors were removed at 4-day intervals after inoculation and the metastasis to the lung was quantified 2 weeks after the surgical excision of primary tumor. Although lung metastasis was not apparent within 8 days after inoculation, tumor nodules were observed in the lung when primary tumors were removed 12 or more days after inoculation (Fig. 2).

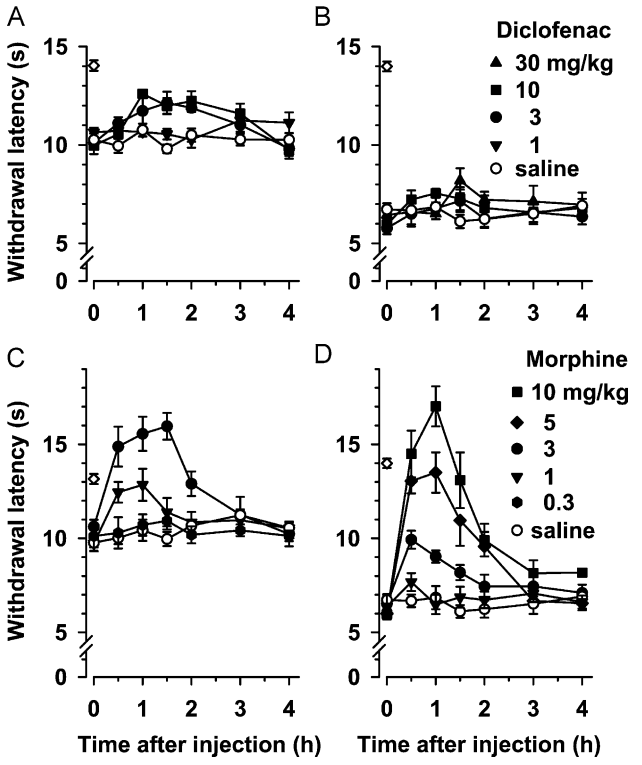

Fig. 3. Effects of diclofenac and morphine on the thermal hyperalgesia of melanoma-inoculated hind paw of the mice. Mice were inoculated with melanoma cells, as described in Fig. 1. (A,B) Diclofenac sodium was injected intraperitoneally on day 8 (A) and day 16 post-inoculation (B). Diclofenac significantly inhibited hyperalgesia on day 8 post-inoculation (RM-ANOVA; main effect, $F(3,27)=4.16$, $P<0.05$; interaction between treatment and time, $F(18,162)=3.49$, $P<0.001$), but not on day 16 post-inoculation. (C,D) Morphine hydrochloride was injected subcutaneously on day 8 (C) and day 16 post-inoculation (D). Morphine significantly inhibited hyperalgesia on day 8 (RM-ANOVA; main effect, $F(3,26)=9.75$, $P<0.001$; interaction between treatment and time, $F(18,156)=6.22$, $P<0.001$) and day 16 post-inoculation (RM-ANOVA; main effect, $F(4,25)=18.2$, $P<0.001$; interaction between treatment and time, $F(24,150)=11.9$, $P<0.001$). Closed symbols, diclofenac or morphine; open circles, saline; open diamonds, contralateral hind paw. Each point represents the mean and S.E.M. of 6–8 animals.

3.2. Antinociceptive effects of diclofenac and morphine

Licking behavior varied from day to day even after day 7 post-inoculation, while thermal and mechanical hyperalgesia and mechanical allodynia were relatively stable especially after day 16 post-inoculation. Mechanical stimulation of melanoma-bearing region elicited vigorous flinching of the hind paw, which was knocked against the wire mesh floor to produce bleeding from the melanoma. Thus, thermal hyperalgesia was most convenient for repeated testing, which brought us to examine effects of analgesics on thermal hyperalgesia. On day 8 post-inoculation (early phase), an intraperitoneal injection of the nonsteroidal anti-inflammatory drug diclofenac sodium (3 and 10 mg/kg) significantly, but partly, inhibited the hyperalgesia, without apparent effects at a dose of 1 mg/kg (Fig. 3A). On day 16 post-inoculation (late phase), the hyperalgesia was no affected by diclofenac at doses of 3–30 mg/kg (Fig. 3B).

On day 8 post-inoculation, subcutaneous morphine at doses of 1 and 3 mg/kg, but not 0.3 mg/kg, produced a dose-dependent inhibition of the thermal hyperalgesia; complete inhibition occurred at doses of 1 and 3 mg/kg (Fig. 3C). On the other hand, on day 16 post-inoculation, morphine at a dose of 1 mg/kg was without effect. The hyperalgesia was dose-dependently inhibited by higher morphine doses of 3–10 mg/kg; the doses of 5 and 10 mg/kg produced complete inhibition of the hyperalgesia (Fig. 3D).

When morphine was given at a subcutaneous dose of 5 mg/kg on day 22 post-inoculation, spontaneous licking of the inoculated hind paw was markedly inhibited; the duration of licking for 1 h after the injection was 135 ± 47,

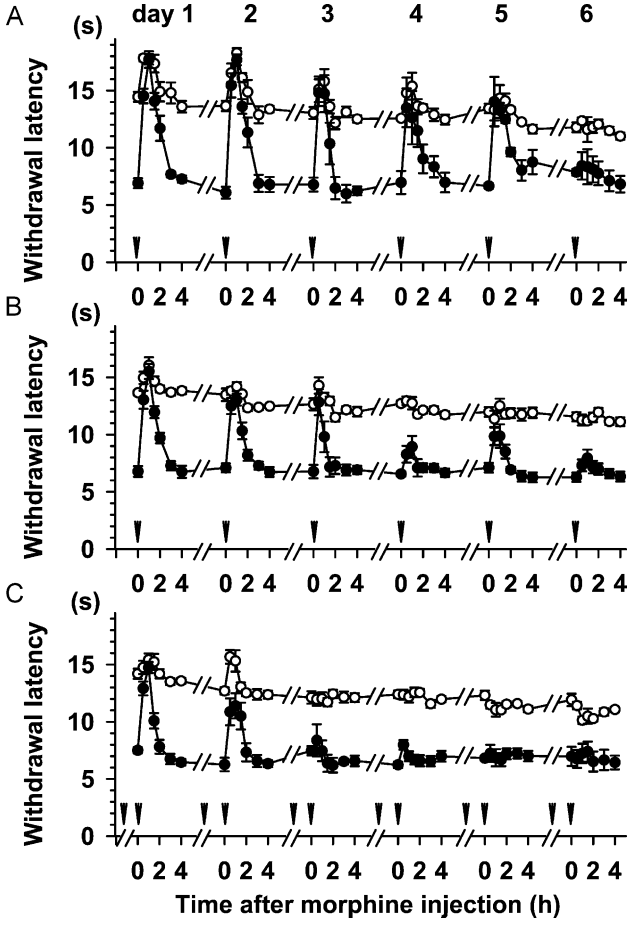

Fig. 4. Effects of repeated injections of morphine on the thermal hyperalgesia of melanoma-inoculated hind paw of the mouse. Mice were inoculated with melanoma cells, as described in Fig. 1. Morphine hydrochloride was administered subcutaneously at doses of 10 mg/kg (A), 5 mg/kg once a day (B) and 5 mg/kg twice a day (C) daily from day 16 post-inoculation. The arrowheads indicate the time of morphine injection. Arrowheads, morphine injection; closed circles, melanoma-inoculated hind paw; open circles, contralateral hind paw. Each point represents the mean and S.E.M. of 5–10 animals.

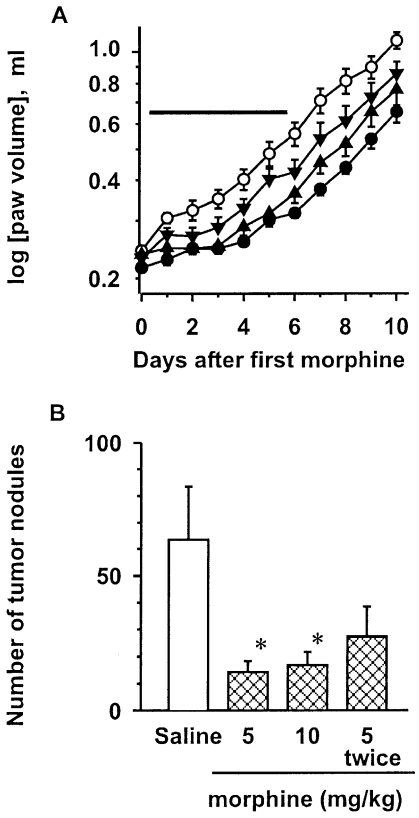

Fig. 5. Suppression of the growth and metastasis of tumor by analgesic doses of morphine. (A) Inhibition of tumor growth by morphine. The mice were inoculated with melanoma cells, as described in Fig. 1. Saline (open circles) and morphine hydrochloride at doses of 5 mg/kg once a day (closed circles), 10 mg/kg once a day (closed up triangles) and 5 mg/kg twice a day (closed down triangles) were administered subcutaneously from day 16 to day 21 post-inoculation (period indicated by the horizontal bar), as shown in Fig. 3. To make the duration of inhibition of tumor growth clear, the volume of the hind paw is plotted in common logarithm. Analysis with RM-ANOVA revealed significant main effect of morphine ($F(3,42)=7.95$, $P<0.001$) and interaction between morphine and time ($F(30,420)=4.75$, $P<0.001$). (B) Inhibition by morphine of the lung metastasis of melanoma cells. Primary tumors of the same mice as (A) was excised on day 25 post-inoculation, and 14 days later, the number of tumor nodules in the lung was counted. * $P<0.05$ compared with saline (Dunnett's test). Each point represents the mean and S.E.M. of 10–12 animals.

45 ± 24 and 176 ± 57 s before morphine, following morphine and on day 23 post-inoculation, respectively.

Daily injections of morphine (5 or 10 mg/kg once a day) apparently inhibited the thermal hyperalgesia for 5 days, but the inhibition almost disappeared on the sixth day (Fig. 4A,B). On the other hand, when morphine (5 mg/kg) was injected twice a day, the antinociception was apparent for only 2 days and, thereafter, disappeared (Fig. 4C).

3.3. Effects of morphine and sciatic neurectomy on tumor growth and metastasis

In this series of experiments, we examined the effects of consecutive administration of morphine for 6 days (as shown in Fig. 4) on tumor growth and metastasis. Tumor growth (i.e., increase in the volume of the inoculated hind paw) was significantly ($P<0.001$ for main effect of morphine and interaction between morphine and time) inhibited by analgesic doses of morphine examined (Fig. 5A). Morphine (5 and 10 mg/kg once a day) apparently inhibited the increase until 3–4 days after the start of administration, while morphine (5 mg/kg twice a day) showed apparent inhibition for 2 days (Fig. 5A). Although there were no significant differences between morphine doses, the order of inhibition was 5 mg/kg once a day, 10 mg/kg once a day and 5 mg/kg twice a day.

Morphine (5 and 10 mg/kg once a day) significantly decreased the number of tumor nodules in the lung down to 23% and 26% of the control (Fig. 5B). Although the effect of morphine (5 mg/kg twice a day) was not statistically significant, the number of tumor nodules decreased down to 43% of the control (Fig. 5B).

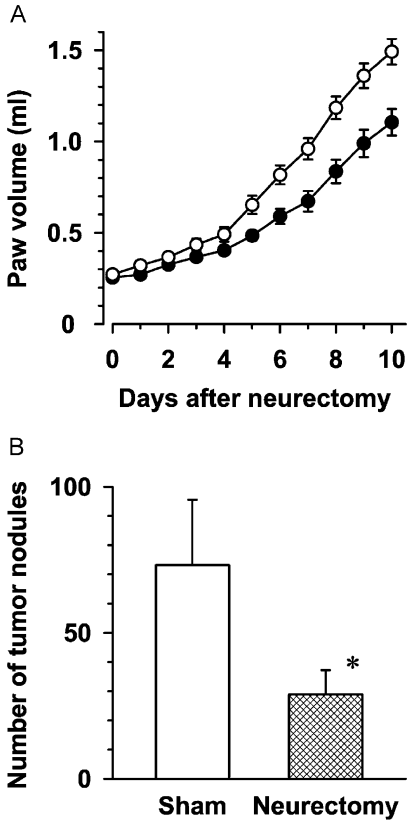

Fig. 6. Suppression of the growth and metastasis of tumor by sciatic neurectomy. (A) Inhibition of tumor growth by neurectomy. Mice were inoculated with melanoma cells, as described in Fig. 1. On day 15 post-inoculation, the mice were given sciatic neurectomy (closed circles) or sham operation (open circles) on the inoculated side. Analysis with RM-ANOVA revealed significant main effect of neurectomy ($F(1,18)=14.45$, $P<0.01$) and interaction between neurectomy and time ($F(9,162)=9.17$, $P<0.001$). (B) Inhibition by sciatic neurectomy of lung metastasis of melanoma cells. The lung metastasis was examined as described in Fig. 4. * $P<0.05$ (Student's t-test). Each point represents the mean and S.E.M. of 9–10 animals.

The neurectomy of the sciatic nerve innervating the inoculated region significantly suppressed the tumor growth (main effect of neurectomy, $P<0.01$; interaction between neurectomy and time, $P<0.001$) and lung metastasis ($P<0.05$) (Fig. 6).

3.4. Effect of morphine on tumor growth in vitro

Morphine at concentrations of 0.35–350 μM produced no apparent inhibition of the proliferation of B16–BL6 melanoma cells, although higher concentration (3500 μM) of morphine showed a marked inhibition ($n=3$ each concentration).

4. Discussion

The orthotopic inoculation of B16–BL6 melanoma cells to C57BL/6 mice induced several types of pain symptoms. Thermal hyperalgesia became apparent between day 7 and day 11 post-inoculation (early phase). From day 12 to day 13 post-inoculation (late phase), the hyperalgesia became marked, and licking behavior (probably due to spontaneous pain) became apparent. These findings taken together indicate that there are two phases, with at least two distinct mechanisms, in pain responses to tumor inoculation. At the early phase (around day 8 post-inoculation), tumor growth just became apparent from the paw volume and no metastasis was observed. On day 8 post-inoculation, although the increase of paw volume was much less than that of carrageenan-treated paw, the decrease of withdrawal latency was similar to each other between tumor inoculation and carrageenan-induced inflammation. Relatively high doses of diclofenac significantly, but partly, inhibited the hyperalgesia on day 8 post-inoculation. Therefore, it is suggested that the early phase of pain response was at least partly due to tumor-induced inflammation, including prostaglandin production.

During the late phase (around day 16 post-inoculation), the increase of paw volume was comparable with that of carrageenan-induced inflammation, while the shortening of the withdrawal latency was much greater than that of carrageenan. Morphine at a dose of 1 mg/kg completely inhibited the hyperalgesia at the early phase but did not affect it at the late phase. These findings suggest the severity of late-phase pain. Although diclofenac was effective against early-phase pain, it was without effects at the late phase, suggesting that prostaglandins do not play an important role in the late-phase pain. At present, the mechanisms of the late-phase pain are unclear. However, the data that metastasis of melanoma cells began at the late phase after inoculation raises the possibility that any factors involved in tumor metastasis are responsible for the late-phase pain, although we do not exclude the involvement of neuropathic component. The other possibility is that any factors such as cytokines, growth factors, etc. produced in and secreted from in vivo tumor mass changing with the tumor growth may be involved in the appearance of cancer pain. In this context, we have found that when naive mice were given an intraplantar injection of the extract of tumor mass isolated from mice with melanoma of the day 16 to day 20 post-inoculation, they showed marked hyperalgesia and spontaneous licking behavior (Zhang et al., 2001). The elucidation of the mechanisms of the late-phase pain may provide a useful basis for the treatment of cancer pain.

One important finding in the present study is that the repeated administration of analgesic doses of morphine at the late phase markedly suppressed the growth and metastasis of tumor cells. Although the present study does not provide direct evidence demonstrating the mechanisms of such suppression, one possible explanation is that the anti-tumor effect of morphine resulted from the relief of cancer pain. Supporting this idea, the blockade of pain signals by sciatic neurectomy also suppressed the tumor growth and metastasis. Since cancer pain was severe at the late phase, it may be a strong stressor for the mouse. Anti-metastatic effect of morphine (5 mg/kg twice a day) was less than that of morphine (5 and 10 mg/kg once a day). This may be associated with the duration of analgesic effects of morphine. We have recently found that psychological stress enhances the liver metastasis of colon tumor, in part by suppressing cellular immunity in mice (Wu et al., 2000). There is evidence suggesting that various stresses promote tumor growth and metastasis (Giraldi et al., 1994; Kanno et al., 1997; Holden et al., 1998; Ben-Eliyahu et al., 1999). The suppression of immune functions such as decrease in natural killer activity was claimed to be involved (Holden et al., 1998; Ben-Eliyahu et al., 1999). Pre- and postoperative administration of an analgesic dose of morphine attenuates the surgery-induced increase in metastasis (Page et al., 1993). In that case, the attenuation was suggested to be caused by prevention of surgery-induced increases in plasma corticosterone concentration (Page et al., 1998). With these findings taken into account, the present results suggest that sufficient relief from pain by medication of morphine in cancer patients is needed to improve quality of life and anti-tumor efficacy. Further studies are needed to determine the mechanisms of morphine-induced inhibition of the growth and metastasis of tumor cells.

Another explanation for anti-tumor effect of morphine is that morphine acts directly to inhibit tumor cell growth. Morphine was reported to inhibit the growth of tumor cells at relatively high concentrations (Ishikawa et al., 1993; Sueoka et al., 1996). In fact, in the present experiments, morphine at an extremely high concentration (3.5 mM) exerted cytotoxic effect on B16–BL6 cells. However, given that morphine was distributed evenly throughout the body, the doses of 5 and 10 mg/kg correspond to about 13 and 26 μM, respectively. Morphine at concentrations up to 350 μM did not affect the growth of B16–BL6 cells in vitro. Therefore, the anti-tumor action of analgesic doses of morphine may not be due to the direct cytotoxic effect.

In summary, we have developed a mouse model of cancer pain, in which cancer pain is easily assessed together with tumor growth and metastasis. Pain became marked at the late phase, when tumor cells began to metastasize, suggesting that metastasis-related factors and/or phenomena are involved in the late-phase pain. Our results also suggest that the relief from cancer pain by medication of morphine prevents tumor growth and metastasis.

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