Enhancement of Tumor Growth by Morphine and Its Possible Mechanism in Mice

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The effect of morphine on tumor growth of EL-4 leukemia in C3H/BL/6 mice and of Sarcoma 180 carcinoma in ddY mice was studied. Local subcutaneous tumor growth was enhanced by morphine (10 mg/kg, s.c.) given daily for 10 d. This effect was inhibited by preadministration of the opioid antagonist naloxone. However, naloxone alone had no significant effect on tumor growth. Morphine also enhanced tumor growth in C3H/BL/6 mice inoculated i.p. with P388 as well as Meth-A cell in Balb/c mice. However, incubation of morphine with cultures of EL-4, P388, MM-46 and Meth-A cells failed to enhance tumor growth. Mice given morphine displayed marked atrophy and reduced cellularity of the spleen and thymus. The humoral response to sheep erythrocytes and Th and B-cell responses to foreign antigens were suppressed, and the lymphocyte proliferative response to T- and B-cell mitogens (concanavalin A and bacterial lipopolysaccharide, respectively) was attenuated. Morphine exerted an inhibitory effect on the immune response which was antagonized by the concomitant administration of naloxone. These data suggest that the enhancement of tumor growth by the administration of morphine is the result of a overall immunosuppressive effect. The significance of the immunomodulatory effect of morphine is discussed in this report.

Keywords morphine; EL-4 leukemia; naloxone; immunosuppression; tumor enhancement

Opioids, such as morphine and their endogeneous peptide counterparts, produce multiple pharmacological effects and exert many physiological functions. There is also increasing evidence that the neural and immune systems are functionally interconnected. An increase in the incidence of infectious disease has been reported in opioid addicts, as well as a reduction in T-lymphocyte number and function. In animal models, opioids can be shown to inhibit lymphocyte proliferative responses, natural killer cell activity, and antibody production. Specific binding sites for morphine and endogeneous opioid peptides are also found on circulating lymphocytes.

In addition, morphine can increase pituitary prolactin and growth hormone secretion, and reduce gonadotropin and thyrotropin secretion. Moreover, it was reported that naloxone given alone to animals can decrease prolactin and growth hormone secretion and that naloxone can increase secretion of gonadotropin, suggesting that the endogeneous opioids participate in control of anterior pituitary hormone function. The development and growth of tumors in animals are dependent on prolactin and ovarian steroids and may be influenced by growth hormone. It has been known that prolongation of survival time and tumor growth retardation by heroin in mice inoculated with tumor cell can be reversed by concomitant administration of naloxone. Naloxone has been demonstrated to prolong survival time and to inhibit tumor growth in mice inoculated with tumor cells. However, the role of morphine in the immune system and in tumor growth in vivo remains unclear. Therefore, we considered it of interest to determine whether administration of morphine could alter tumor growth in mice, a model commonly used in study of opiate tolerance and dependence. Morphine was chosen as the opiate because it is a potent narcotic that is used clinically for the treatment of chronic pain, particularly in cancer patients.

Materials and Methods

Mice and Tumors Inbred male C3H/BL/6 mice, ddY male mice and C3H/He male mice, 5 weeks of age obtained from the Japan SLC in Hamamatsu (Japan), were housed in plastic cages with wood shavings as bedding, and fed a commercial diet and water ad libitum. They were maintained at 25 ± 1 °C and 50 to 55% humidity with a cycle of 12-h fluorescent light/12-h darkness. EL-4 Lymphoma (EL-4) in C3H/BL/6 mice, Sarcoma 180 carcinoma (Sarcoma 180) in ddY mice, MM-46 Mannmary (MM-46) in C3H/He mice, P388 lymphoma (P388) in C3H/BL/6 mice and Meth-A fibrosarcoma (Meth-A) in Balb/c mice, were maintained by weekly passage in our laboratory. Research protocols were approved by the Guideline for Animal Experimentation for Tohoku College of Pharmacy.

EL-4, P388, MM-46 and Meth-A for culture were maintained by stationary culture in RPMI-1640 medium supplemented with 4% fetal bovine serum.

Chemicals Morphine hydrochloride (morphine) and naloxone hydrochloride (naloxone) were obtained from Sankyo Co., Ltd. (Tokyo, Japan) and Sigma (St. Louis, MO, U.S.A.), respectively. These chemicals were dissolved in saline prior to use, and all drug injections were administered subcutaneously. Lipopolysaccharide (LPS; Westphal extraction) from E. coli 020. B6 was obtained from Difco Laboratories (Detroit, MI, U.S.A.). Citrated sheep red blood cells (SRBC) were received from Kyokuto Chemicals (Tokyo, Japan).

Tumor Models and Antitumor Activity Experiments To determine the effect of morphine on the growth of tumor cells, the following experimental models were used. (a) Solid-type tumor cells were examined in Sarcoma 180 in ddY mice and EL-4 leukemia (EL-4) in C3H/BL/6 mice. Sarcoma 180 was inoculated by s.c. injection of 3 x 10⁵ cells into the right thigh. EL-4 was transplanted by s.c. injection of 3 x 10⁵ cells into the right axilla of groups of 8-12 mice which were then given an injection of morphine (10 mg/kg, s.c.), with or without naloxone (1 mg/kg, s.c.), once daily for 10 d, starting 24 h after inoculation with tumor cells. Sixteen or 23 d after Sarcoma 180 and EL-4 tumor cell inoculation, mice were sacrificed, and the tumors were removed and weighed. Data are expressed as the means ± S.E. of the tumor weight (g). (b) Ascites-type tumor cells were examined in P388 (P388) in C3H/BL/6 mice and Meth-A in Balb/c mice. Mice were inoculated intraperitoneally with P388 or Meth-A (10⁴ or 10⁵ cells/mouse, respectively). Morphine was injected once daily for 10 d, starting 24 h after inoculation with tumor cells. Each group of ten mice was observed daily, and terminated at 21 d (P388) and 29 d (Meth-A).

Microcystostasis Assay (MTT Assay) A total of 4 experimental tumor cell lines, EL-4, P388, Meth-A and MM-46, were studied. A volume of 100 μl medium containing 3 x 10⁴ viable cells were plated per well into 96-well microtitre plates. Cells were allowed to attach to the plates for 24 h at 37 °C in a 5% CO₂ atmosphere. Morphine was dissolved in sterile saline at 1 mg/ml was used after sterile filtration through 0.22-μm Millipore filters. Drug dilutions were prepared in culture medium (range, 100—800 μg/ml). Medium was removed from the cells and test dilutions were added in 100 μl

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The effect of morphine on the growth of Sarcoma 180 and EL-4 leukemia (EL-4) were examined in a solid-type tumor in mice. Sarcoma 180 tumor cells, $3 \times 10^5 - 3 \times 10^6$ were inoculated by s.c. injection into the right thigh of groups of 8-12 12-d-old mice. EL-4 was transplanted by s.c. injection of $3 \times 10^5 - 3 \times 10^6$ tumor cells into the right axilla of groups of 8-12 C57BL/6 mice. The animals received injections of morphine (10 mg/kg, s.c., closed cycle) or saline (open cycle) once daily for 10 d, starting 24 h after tumor cell inoculation. Sixteen or 23 d after tumor cell inoculation, mice were sacrificed, and the tumors were removed and weighed. Data are expressed as the mean ± S.E. of tumor weights (g). Data were analyzed by Student's t-test; a) $p < 0.05$ with respect to the control group.

Fig. 1. The Effect of Morphine on the Growth of Sarcoma 180 and EL-4 Leukemia in Mice

Tissue Preparation Twenty-four hours after the last morphine injection, C57BL/6 mice were sacrificed by cervical dislocation. Body weight was recorded and the thymus and spleen were removed aseptically for weighing. Adrenal glands were also removed and weighed in some experiments. Relative spleen, thymus and adrenal gland weights were calculated by dividing the organ weight (mg) by the body weight (g). Single cell suspensions were prepared by teasing with sharp forceps in Petri dishes containing RPMI 1640 culture medium (RMPI, Nakarai Inc., Kyoto, Japan) and these were washed three times in RPMI by centrifugation. The washed spleen cells were assayed for antibody-forming cells and lymphocyte proliferative response.

Antibody Response to SRBC and LPS Mice were immunized by an intraperitoneal injection of 0.2 ml of washed sheep red blood cells (SRBC) or LPS. Four days later splenic antibody-secreting (plaque forming) cells were enumerated using the Jerne hemolytic plaque technique. Indicator red cells prepared according to the method of Moller were used for the preparation of LPS SRBC.

Lymphocyte Proliferative Response The spleen cells were washed in an ammonium chloride buffer (155 mM NH₄Cl; 7 mM KHCO₃; 0.1 mM EDTA) for 90 s to hemolyze red blood cells. The spleen cells were then resuspended to give a concentration of $2.5 \times 10^5$ cells/ml in RPMI containing 5% fetal calf serum, 12 mM HEPES buffer and 0.05 mg/ml gentamycin. One hundred microliters of the cell suspension was added to the appropriate well of a plastic microculture plate ( Falcon Plastics, Oxnard, CA, U.S.A.). Each well contained 100 ml of medium, with or without mitogen. A range of concentrations of each mitogen was tested for splenic lymphocyte stimulation. The range of final concentrations in the cultures was 0.4 to 40.0 mg/ml for concanavalin A (Con A; Pharmacia Fine Chemicals, Sweden) and 0.2 to 10 mg/ml for LPS. The cells were cultured at 37 C in a humidified atmosphere with 5% CO₂ and 95% air for 72 h. A reverse c100 ml of 0.5%Ci of methyl-[3H]thymidine (New England Nuclear, Boston, MA, U.S.A.) was added to each well 24 h before the culture was terminated. At the end of the culture period, cells were harvested on glass-fibre filters using a cell harvester. After drying, radioactivity was determined by counting in a Packard Liquid Scintillation Counter. Each cell culture was performed in triplicate and the response to the optimal dose of each mitogen was expressed as the net (difference between the experimental value and control value) cpm per culture.

Results

Effects of Morphine on Tumor Growth in Mice The effect of morphine on tumor growth was examined in two experimental models. In the early solid-type Sarcoma 180 and EL-4 tumor cells, at $3 \times 10^5$ cells/mouse, morphine did not appreciably influence tumor growth. However, at lower transplantation rates ($3 \times 10^3 - 10^6$ cells/mouse), morphine effectively increased the tumor weight compared with controls (Fig. 1). To determine whether morphine-induced tumor growth development was specific, naloxone was injected prior to administration of morphine. The effect of naloxone alone, or in combination with morphine, on the
tumor growth is shown in Fig. 2. Naloxone inhibited the development of tumor growth produced by morphine, but did not suppress normal tumor growth. As the effect of morphine was antagonized by naloxone, it is suggested that morphine activates tumor through an opioid receptor in tumor cells. At higher doses of naloxone (8 mg/kg, s.c.) it should exert an antitumor effect.

When a similar treatment schedule was used, the same influence of morphine on the tumor growth of ascites-type P388 or Meth-A-bearing mice was observed (Fig. 3).

To determine the mechanism by which morphine alters tumor growth in the mice, an experiment was conducted in which morphine was added in vitro to tumor cells. The purpose of the experiment was to determine if morphine could directly enhance tumor growth. The results of the in vitro effects of morphine on the growth of EL-4, P388, Meth-A and MM-46 cells are presented in Table I. When 5 x 10^5 confluent cells of each type were incubated in the presence of various morphine concentrations, the cytotoxic effect of morphine on these cell lines was dose-dependent. The concentrations of morphine required for 50% inhibition of cell growth at 48 or 72 h were 170 or 210 µg/ml for EL-4, 460 or 440 µg/ml for P388, 610 or 560 µg/ml for Meth-A and 290 or 290 µg/ml for MM-46, respectively.

**Body and Tissue Weight** In order to examine the effects on body, spleen, thymus and adrenals weights, morphine was injected i.p. for 10 d. Although there were no significant differences between the body weights of control and morphine-treated mice, a significant decrease in the relative spleen and thymus weights (spleen or thymus/body weight) was observed in morphine-treated animals (Fig. 4). No differences were noted in the relative adrenals weights of morphine-treated mice.

**Plaque-Forming Cell (PFC) Response** To determine the mechanism by which morphine alters tumor growth in mice, we investigated the immunomodulatory effect (two-type immune phase of plaque-forming cell response and lymphoproliferative response) of morphine in mice. Despite extensive pharmacological studies on morphine, there is little information on immunotoxicity in chronically morphine-treated mice. The effect of morphine on the antigen-specific PFC response of animals immunized with either SRBC, a macrophage-dependent T-cell antigen, or LPS, a macrophage-independent B-cell antigen, was compared. It can be seen from Fig. 5 that morphine had an immunosuppressive effect on the anti-SRBC and anti-LPS response. Naloxone (1 mg/kg, s.c.) had no effect on either PFC response. However, when naloxone was administered 30 min before morphine, it antagonized the

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**Fig. 3.** The Effect of Morphine on the Growth of P388 Leukemia and Meth-A Fibrosarcoma in Mice

The effects of morphine on the growth of P388 leukemia (P 388) in C57BL/6 mice and Meth-A fibrosarcoma (Meth-A) in Balb/c mice were examined against ascites-type tumors in mice. Mice were inoculated intraperitoneally with P 388 cells or Meth-A cells (10^4 or 10^5 cells/mouse, respectively). Morphine (10 mg/kg, s.c., closed circle) or saline (open circle) injected, once daily for 10 d, starting 24 h after inoculation with tumor cells. Deaths were monitored daily and the experiment was terminated at 21 d for P 388 and at 29 d for Meth-A.

**Fig. 4.** Effect of Morphine on Mouse Spleen, Thymus and Adrenals Weights

C57BL/6 mice were injected with morphine (10 mg/kg, s.c., dotted column), or saline (shaded column), once a day for 10 d. Twenty-four hours after the final injection of morphine, mice were sacrificed, and the whole body and organs were weighed. Each bar represents mean ± S.E. Data were analyzed by Student's t-test: *p* < 0.05 with respect to the control.

**Table I.** The Effect of Morphine on the Tumor Growth in Vitro

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC_{50} (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>EL-4</td>
<td>170</td>
</tr>
<tr>
<td>P388</td>
<td>460</td>
</tr>
<tr>
<td>Meth-A</td>
<td>610</td>
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<tr>
<td>MM-46</td>
<td>290</td>
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Each cell line (5 x 10^5 cells/well) was cultured in a 96-well microtest plate for 48 or 72 h at 37°C. Assays were done in triplicate. The experimental procedures and conditions are as described in Materials and Methods.
inhibitory effect of morphine on both these parameters.

**Lymphocyte Proliferative Response** Morphine inhibited the lymphoproliferative response in C57BL/6 spleen cells. Furthermore, significant suppression of the lymphocyte proliferative response to the T-cell mitogen Con A and the B-cell mitogen LPS was observed in morphine-treated mice (Fig. 6). Naloxone significantly inhibited the lymphoproliferative response to native cells but naloxone did not significantly affect the lymphoproliferative responses to either mitogen. The effects of the administration of naloxone upon this effect were puzzling. However, when naloxone was administered 30 min before morphine, it antagonized the inhibitory effect of morphine to native cell and both mitogens (data not shown).

**Discussion**

The present results demonstrate that morphine can significantly alter tumor growth. The EL-4 and Sarcoma 180 tumor cells have been widely used as in vivo models for the study of human neoplasm, particularly with regard to the screening of therapeutic agents. There was a significant increase in tumor weight in morphine-treated (10 mg/kg s.c., daily, 10 d) mice inoculated with EL-4 and Sarcoma 180 tumor cells. The magnitude of this enhancement effect of morphine inversely dependent on the number of cells inoculated. The mechanism of this cell number-dependent effect by morphine against the tumor growth can not be explained at present. Moreover, morphine diminished the survival time of C57BL/6 mice inoculated with P388 tumor cell (10⁶) and Balb/c mice inoculated with Meth-A tumor cell (10⁶). This is in contrast to the effects of antagonists of opioid drugs as naloxone and naltrexone¹, and opioid peptides which inhibit the growth of mammary cancers. Morphine enhancement of EL-4 lymphoma growth was blocked by the continuous administration of naloxone, providing evidence that this enhanced tumor growth is mediated by opioid receptors. In other studies, no enhancement of tumor growth by morphine was observed directly on EL-4, P388, Meth-A and MM-46 tumor cells. Morphine treatment thus may be

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**Fig. 5. Effect of Morphine on the Splenic Plaque-Forming Cell Response**

C₅7BL/6 mice were immunized with 5 x 10⁴ sheep red blood cells (SRBC) or 10 µg LPS which were administered intraperitoneally. Spleen cells from the immunized animals were harvested for plaque-forming cell assay 4 d after the injection of antigen. Morphine (10 mg/kg, s.c.) (M) was injected, once a day for 10 d, and naloxone (1 mg/kg, s.c.) (Nax) was injected 30 min before the daily morphine administration. The final injection of morphine was given 24 h prior to harvesting spleen cells. Each point represents the mean ± S.E. of five to seven animals. Data were analyzed by Student's t-test; a) p<0.05 with respect to control.

**Fig. 6. Effect of Morphine on the Lymphocyte Proliferative Response**

C₅7BL/6 mice were given morphine (10 mg/kg, s.c.) (M) or saline once a day for 10 d and naloxone (1 mg/kg, s.c.) (Nax) 30 min before the daily morphine administration. Splen cells of the animals were harvested for lymphocyte proliferative response 24 h after the final morphine injection. Lymphocyte proliferative responses to native or stimulated cells (cell suspension with 1 µg Con A or 0.5 µg LPS to each well) were assessed by the uptake of methyl-³H-thymidine by the lymphocytes. Each point represents the mean ± S.E. of five to six animals. Data were analyzed by Student's t-test; a) p<0.05 with respect to control and b) p<0.05 with respect to Con A or LPS alone.
have a broad effect on the tumor growth in mice inoculated with tumor cell. Since the present study was performed in the mouse, a human study is needed to resolve the question of a possible interaction in man.

The mechanism by which morphine increases tumor growth \textit{in vivo} is not clear. Opioids may influence tumor growth by modulating the immune system. Many tumors are significantly affected by immune mechanisms and there is growing evidence that opioids affect immune system function. Hazum and colleagues\textsuperscript{22} have reported a specific binding site for opioids on mitogen-transformed cultured human lymphocytes. Other \textit{in vitro} studies have demonstrated that opiates or opiate antagonists can regulate mitogen-induced lymphocyte proliferation.\textsuperscript{5,6,11,12} Moreover, it has been demonstrated that the cytotoxic activity of natural killer (NK) cells, components of the immune system thought to be involved in cancer surveillance, is markedly suppressed following administration of opioids and this suppression was prevented by pretreatment with naltraxone.\textsuperscript{23} The present study was designed to elucidate the role of the immune response in the modulation of tumorigenicity by morphine. Morphine (10mg/kg, s.c.) displayed marked atropy and an attenuated lymphocyte proliferative response to T- and B-cell mitogens (Con A and LPS, respectively). Also, the antigen-specific PFC response of animals immunized with either SRBC, a macrophage-dependent T-cell antigen, or LPS, a macrophage-independent B-cell antigen, agrees with findings showing suppression of other immunologic endpoints and increased susceptibility to infection after chronic exposure to morphine.\textsuperscript{7,24,25} It is known that, to elicit an antibody response to a T-cell-dependent antigen such as SRBC, the participation of macrophages and T cells in the initial antigen processing and presentation is required. Therefore, macrophages and T lymphocytes can be considered as possible targets for morphine. Naloxone interfered with the immunosuppressive effect of morphine. Because morphine can diminish immune functions, the host immune response to tumor cells may be diminished. Therefore, a better understanding of the physiological mechanisms of morphine-induced immunosuppression and the relationship between the immune system and the phenomena of opioid tolerance and dependence is needed to understand more fully this complex relationship.

Another possible explanation for the morphine-induced enhancement of tumor growth could be due to a change in the milieu. Administration of opiates stimulates the release of other hormones, including prolactin, growth hormone, and insulin\textsuperscript{26} and it is possible that these hormones may affect tumor growth. Of interest, is the fact that tumor cells can generate hormone-like substances that can influence hormone release by host cells, potentially creating a hormonal milieu that would favor tumor growth.\textsuperscript{27} This "growth self-inciting" phenomenon in tumors has been described, with B 16 melanoma as the model.\textsuperscript{28}

Direct enhancement of tumor growth is another possible explanation. However, the growth-inhibiting properties of opiates in cultured tissues are well known and retarded tumor cell growth in the presence of opiates and endorphin can be blocked by coadministration of naloxone.\textsuperscript{29} Moreover, in the present study, morphine inhibited tumor cell growth in cultures. Therefore, direct action of morphine is unlikely to play an important role.

Our results indicate that morphine can significantly alter the growth of experimental tumor cells. Our evidence suggests that morphine and opiate receptors play a role in tumor growth. The underlying explanation for the ability of morphine to alter tumor growth in mice is not readily apparent. Further studies are needed to clarify the mechanism of the development of EL-4 lymphoma growth induced by morphine.

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References