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Preliminarily Novel Effect of Morphine on the Tumor Growth in C57BL/6J Mice

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Abstract

The influence of morphine on tumor growth of EL-4 leukemia in C57BL/6J mice was studied. Local subcutaneous tumor growth was enhanced by morphine (10 mg/kg, *s.c.*) daily for 10 days. 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 C57BL/6 mice inoculated i.p. with P388. Furthermore, morphine was found to diminish cisplatin survival rates compared to cisplatin alone. However, incubation of morphine with in cultures of EL-4 and P388 cells did not enhance tumor growth. C57BL/6J mice given morphine displayed marked atrophy and reduced cellularity of the spleen and thymus. The intensity of sheep red blood cells (SRBC)-induced delayed type hypersensitivity in the morphine-treated mice and sensitized with 10^8 SRBC decreased. 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 comprehensively immunosuppresive effect in the mouse and a human study is needed to resolve the quensiton of possible interaction in mice. The meaning of the immunomodulatory effect of morphine is discussed in this report.

Keywords-morphine ; EL-4 leukemia ; P388 leukemia ; naloxone ; mice

Introduction

It is well known that opioids, such as morphine and their endogenous peptide counterparts, produce multiple pharmacological effects and subserve many physiological functions[1]. In addition to their well characterized effects on the immunological response[2~6]. In a recent communication[7,8], we showed that the administration of morphine caused a immunosuppression. The immunosuppression by the drugs will reduce not only immunological defense functions against microorganisms but also immunological resistance against tumors.

Since development and growth of tumors in animals may be influenced by immunity of tumor-bearing host, additional evidence that opioids play a role in tumorigenicity derives

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from phamacological studies showing that opiate antagonists prolong survival time of mice implanted subcutaneously with neuroblastoma cells[9~11], we considered it of interest to determine whether consecutive administration of morphine could alter tumor growth in mice, a model commonly used in the study of opiate tolerance and dependence[12]. Morphine was chosen as the opiate because it is a potent narcotic that is used clinically for the treatment of chronic pain, particularly in terminally ill cancer patients.

Materials and Methods

Mice and tumor Male C57BL/6 mice weighing about 22 g (6 to 7 week-old) at the time of the experiments were obtained from Japan SLC, Inc., Hamamatsu ; and they were housed on a light-dark cycle (light on 6-18 hr) at a constant temperature (25±1°C) and humidity (50 to 55%) for three days before the experiments.

EL-4 Lymphoma (EL-4) and P388 lymphoma (P388) in C57BL/6 mice maintained by weekly passage in our laboratory were used. EL-4 and P388 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 Pharmaceutical Co. Ltd. (Tokyo, Japan) and Sigma (St. Louis, MO, USA), respectively. These chemicals were dissolved in saline prior to use, and all drug injections were administered subcutaneously. Cisplatin injection, 10 mg/vial (Briplatin) was purchased from Bristol Meyers Co. (Tokyo, Japan). Cisplatin were dissolved in sterile saline to obtain solutions of 0.5 mg/ml. This was done immediately prior to the administration of single i.p. injection to mice. 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 treatment on the growth of tumor cells, the following experimental models were used. (a) Solid-type tumor cells were examined in EL-4 leukemia (EL-4) in C57BL/6 mice. EL-4 was transplanted by s.c. injection of $3\times10^5-3\times10^6$ cells into the right axilla of group 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 days, starting 24 hour after inoculation with tumor cells. Twentythree days after 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 inhibitory percentage compared with the control value. (b) Ascites-type tumor cells were examined in P388 leukemia (P388) in C57BL/6 mice. Mice were inoculated intraperitoneally with P388 (10^5 cells/mouse). Morphine was injected once daily for 10 days, starting 24 hour after inoculation with tumor cells. Cisplatin was injected 24 hr after tumor transplantation. Each group of the mice were observed daily, and terminated at 21 days.

Microcytostasis assay (MTT assay)[13] A total of 100 μl medium containing 3×10^5 viable cells were plated per well into 96-well microtitre plates. Morphine dissolved in sterile saline at 1 mg/ml and drug dilutions were prepared in culture medium. Tests were carried out with ten control wells containing cells in the absence of drugs five wells for each test

dilution. Cells were exposed to the drug for 72 h at 37°C in 5% CO_2. After the 48 and 72 h recovery periods following drug exposure, medium was aspirated from each well and 100 μl of a 1 : 10 dilution of MTT (3, 4, 5-dimethylthiazole-2, 5-diphenyltetrazolium bromide, M-2128 ; Sigma Chemical Co., St. Louis, Mo, USA) (5 mg/ml) was added to each well, followed by a 4-hr incubation at 37°C. After incubation, medium was aspirated from each well, 100 μl 0.04 N HCl in 10% dimethylsulfoxide was added well and the plates were agitated on a plate shaker for 20 min at room temperature, then read spectrophotometrically using wavelength filters (550 nm) with a Bio-Tek EL310ElA reader. The concentration of drug causing 50% inhibition of cell growth (IC_{50}) was determined for each line.

Tissue weight One day after the last morphine injection, mice were sacrificed by cervical dislocation. Body weight was recorded and the thymus and spleen were removed asptically 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).

SRBC-induced delayed type hypersensitivity (SRBC-DTH) Mice were sensitized by an subcutaneous injection of an appropriate number of SRBC/40 μl into their left hind footpads and then challenged by an subcutaneous injection of 10^8/40 μl SRBC into their right hind footpads 5 days after the sensitization. The SRBC-DTH intensity was evaluate as the difference in volumes of the left and right footpads measured 24 hr after the challenge by volume meter (MK-550, Muromachi Instruments Co., Ltd., Tokyo, Japan).

Statistical analysis Differences between experimental and control animals were evaluated for significant differences by Student's t-test. Significance was ascribed if $P<0.05$.

Results

Effects of Morphine on Tumor growth in mice

The effect of morphine on the tumor growth was examined in two experimental models. In the early solid-type EL-4 tumor cells, morphine was tested by *s.c.* administration once daily for 10 days starting 24 hours after transplantation of tumor cells ($3\times10^5-3\times10^6$ cells/mouse). At 3×10^6 cells/mouse, morphine did not appreciably influence tumor growth. However, at lower transplantation rates, morphine more effectively increased the tumor weight compared with controls administered with saline (Fig. 1).

To determine whether morphine-induced tumor growth development could be specifically inhibited with naloxone, naloxone were injected prior to administration of morphine. The effect of naloxone alone or in combination with morphineon the tumor growth is shown in Fig. 2. Morphine (10 mg/kg) or naloxone (1 mg/kg) was given once daily for 10 days. In the group that received naloxone plus morphine, naloxone was injected 30 min prior to administration of morphine. Naloxone inhibited the development of tumor growth produced by morphine, but did not suppress base tumor growth. As the effect of morphine was antagonized by naloxone, it is suggested that morphine activates the tumor system through an opioid receptor in effector cells. At higher doses of naloxone (8 mg/kg, *s.c.*) there was

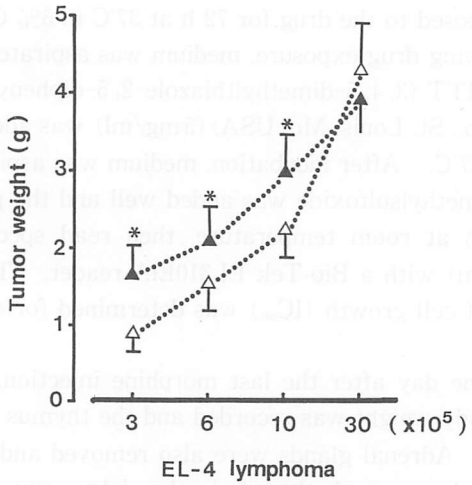

Fig. 1 The effect of morphine on the growth of EL-4 leukemia in mice

The effect of morphine on the growth of EL-4 leukemia (EL-4) were examined in a solid-type tumor in mice. EL-4 was transplated by *s.c.* injection of $3\times10^5-3\times10^6$ 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 mark) or saline (open mark) once daily for 10 days starting 24 hr after tumor cell inoculation. Twenty-three days after tumor cell inoculation, mice were sacrificed, and the tumors were removed and weighed. Data are expressed as the means±S.E. of tumor weights (g). Data were analyzed by Student's t-test ; *, $P<0.05$ with respect to the control group.

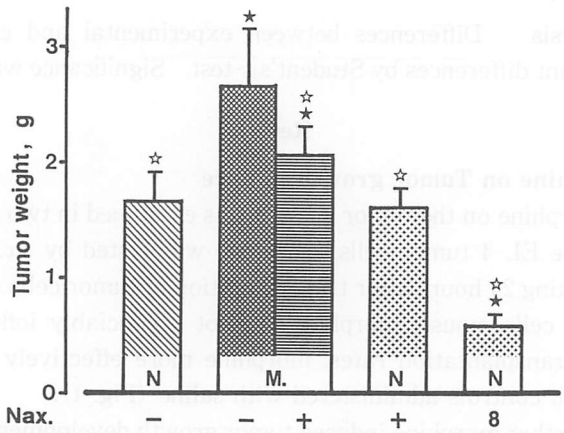

Fig. 2 Effect of naloxone on the morphine-induced modulation of tumor growth in mice

Groups of 8-10 animals were used in each experiment. EL-4 was transplanted by *s.c.* injection of 6×10^5 tumor cells into the right axilla of C57BL/6 mice, then morphine (M) or saline (N) were injected once a day for 10 days 24 hr after inoculation with tumor cells. Naloxone (1 ; +or 8 mg/kg, *s.c.*) (Nax) was injected 30 min before the daily morphine or saline (−) administration. On day 23 after the tumor inoculation, mice were sacrificed and the tumors were removed and weighed. Data are expressed as the means±S.E. of tumor weights (g). Data were analyzed by Student's t-test ; ★, $P<0.05$ with respect to control and ☆, $P<0.05$ with respect to morphine alone.

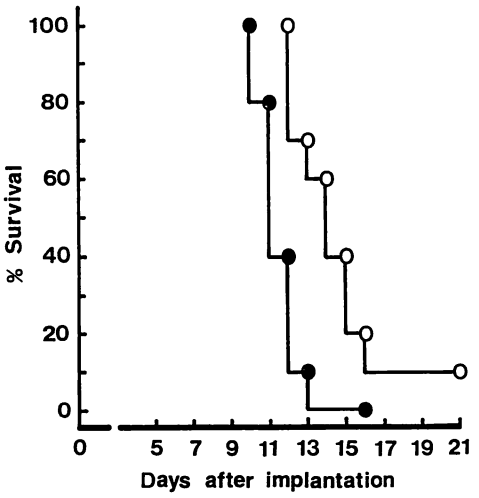

Fig. 3 The effect of morphine on the growth of P388 leukemia in mice

The effects of morphine on the growth of P388 leukemia (P388) in C57BL/6 mice was examined against ascites-type tumors in mice. Mice were inoculated intraperitoneally with P388 cells (10^4 cells/mouse). Morphine (10 mg/kg, *s.c.*, closed circle) or saline (open circle) injected once daily for 10 days from 24 hr after inoculation with tumor cells. Lethality was observed daily and the experiment was terminated at 21 days.

Table 1 The effect of morphine on the tumor growth in vitro

Cell lines	IC_{50} (μg/ml)	
	48 hr	72 hr
EL-4	170	210
P388	460	440

Each cell line (5×10^3 cells/well) was cultured in a 96-well microtest plate for 48 or 72 hr at 37°C. Assays were done in triplicate. The experimental procedures and conditions are as described in "Materials and Methods".

an antitumor effect.

When a similar treatment schedule was used, the same influence of morphine on the tumor growth of ascites-type P388-bearing mice was observed (Fig. 3). On the other hand, the median day of survival for the combination of morphine and cisplatin (8 mg/kg, *i.p.*) was 12.6 days compared to 15.6 days for cisplatin alone. Namely, morphine at a dose of 10 mg/kg with cisplatin appeared to reduce the rate of survival compared to cisplatin alone.

The results of the *in vitro* effects of morphine on the growth of EL-4 and P388 cells are presented in Table 1. When 5×10^3 confluent cells of each type were incubated in the presence of various morphine concentrations. The cytotoxic effect was dose-dependent manner. The concentrations of morphine required for 50% inhibition of the cell growth at 48 or 72 hr were 170 or 210 μg/ml for EL-4 and 460 or 440 μg/ml for P388, respectively.

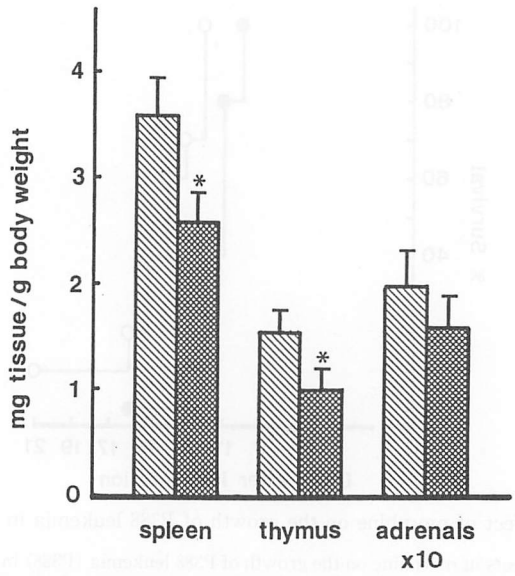

Fig. 4 Effect of morphine on mouse spleen, thymus and adrenal weights

C57BL/6 mice were injected with morphine (10 mg/kg, *s.c.*, dotted column), or saline (shaded column) once a day for 10 days. Twentyfour 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.

Weight

In order to examine the effects on body, spleen, thymus and adrenal weights, morphine was injected *i.p.* for 10 days. There were no significant differences between the body weights of control and morphine-treated mice. A significant depression of relative (spleen or thymus/body weight) spleen and thymus weights was observed in morphine-treated animals (Fig. 4). No differences were noted in the relative adrenal weights of morphine-treated mice.

SRBC-induced delayed type hypersensitivity response

To determine mechanistically how morphine alters tumor growth in mice, we investigated the immunomodulatory effect (immune phase of SRBC-induced delayed type hypersensitivity) of morphine in mice. Despite extensive pharmacological studies on morphine, there is little known about immunotoxicity in successively morphine-treated mice. The purpose of the experiment was to determine if morphine could exert a host-mediated effect of tumor growth.

As preliminary experiment, mice were sensitized with varying doses of $1\times10^5-5\times10^8$ SRBC and then challenged with 10^8 SRBC. Some of the mice were given cyclophosphamide (150 mg/kg, *i.p.*) at 3 days before the sensitization. The SRBC-DTH intensity in the cyclophosphamide non-treated control mice increased dependently on the SRBC dose for sensitization, and it reached a peak by sensitization with 10^8 SRBC. The intensity in mice

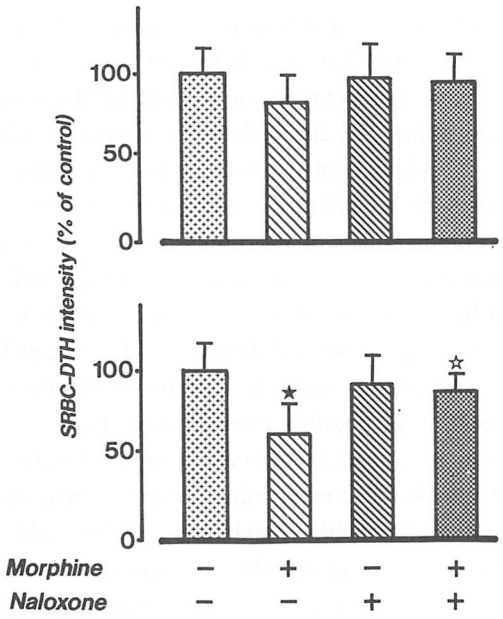

Fig. 5 Effect of morphine on sheep red blood cells-induced delayed type hyperesensitivity in mice

Mice were sensitized with 5×10^5 or 1×10^8 SRBC, *s.c.* into the left hind foodpad and then challenged 5 days later by an *s.c.* injection of 10^8 SRBC into the right hind foodpad. Morphine was given *s.c.* for 5 days starting the day of sensitization. The delayed type hypersensitivity intensity was evaluated by the increase of foodpad volume 24 hr after the challenge. Data represent means±S.E. of 10 or 12 mice. Data were analyzed by Student's t-test ; ★, $P<0.05$ with respect to control and ☆, $P<0.05$ with respect to morphine alone.

treated with cyclophosphamide and sensitized with 10^5 or 10^6 SRBC was not different from that of the control mice. However, in mice treated with cyclophosphamide and immunized by 10^7 or more SRBC, the intensity was 1.8-2.2 times that of the control mice. Therefore, in the subsequent studies, doses of 5×10^5 and 10^8 SRBC were used for the sensitization, and the respective responses were referred to as 5×10^5 SRBC-DTH and 10^8 SRBC-DTH, respectively.

Morphine was given to mice for 5 consecutive days starting the day of sensitization. Morphine showed a tendency to suppress 5×10^5 SRBC-DTH and suppressed 10^8 SRBC-DTH significantly (Fig. 5).

Naloxone (1 mg/kg, *s.c.*) had no effect on SRBC-DTH response. However, when naloxone was administered 30 min before morphine, it antagonized the inhibitory effect of morphine on the 10^8 SRBC-DTH but did not affect 5×10^5 SRBC-DTH.

Discussion

The present results clearly demonstrate that morphine can significantly alter the tumor growth. The EL-4 and P388 lymphoma tumor cells have been widely used as an *in vivo* model for the study of human neoplasia, particularly in regard to the screening of therapeutic

regemens. A significant increase in tumor weight of morphine treated (10 mg/kg *i.p.*, daily, 10 days) mice was seen in mice inoculated with EL-4 tumor cells. The magnitude of the enhancement effect of morphine was contrary dependent on the number of cells utilized for inoculation. Moreover, morphine can diminish survival time of C57BL/6 mice inoculated with P388 tumor cell (10^4). This is in contrast to the effects of antagonist of opioid drugs and endogenous opioid peptides such as naloxone and naltrexone[9,14] which inhibit growth of mammary cancers. And enhancement of morphine against EL-4 Lymphoma growth was blocked by continuous administration of naloxone, providing evidence that this enhanced tumor growth is mediated by opioid receptor. In other studies, no enhancement effect of tumor growth of the morphine was observed directly on EL-4 and P388 tumor cells, which was inhibited in cultures. The present study was performed in the mouse. A human study is needed to resolve the question of possible interaction in man.

The mechanism by which morphine increase tumor growth in vivo are not clear. Recently, we reported that morphine showed enhancement of Sarcoma 180 tumor growth by its immunosuppressive effect, and morphine had host-dependent enhancement effect of tumor growth. Opioids may influence tumor growth by modulating activity of the immune system. It is generally accepted that T lymphocytes primed tumor-associated antigens play an important role in host defense mechanisms against tumors[15~17] in addition to the involvement of some other effector mechanisms including natural killer (NK) cells[18], antibody dependent cell-mediated cytotoxicity[19] and cytotoxic antibodies[20]. It has been also pointed out that not only Lyt-1^-2^+ T cells[21,22] including cytotoxic T lymphocytes but also Lyt-1^+2^- ($L3T4^+$) T cells[21~24] including delayed type hypersensitivity effector cells and amplifier/helper cells play a role in the host defense mechanisms against tumors.

The present study was designed to elucidate the role of immune response in the modulation of tumorigenicity by morphine. In the present experiment mice given a consecutive injection of morphine (10 mg/kg, *i.p.*) suppressed SRBC-DTH and thymus weight. It seems that the suppressive effect of morphine on SRBC-DTH and thymus weight was relatively strong. Also, SRBC-DTH agrees with other findings showing suppression of other immunologic endpoints and increased susceptibility to infection after chronic exposure to morphine[25,26]. It is well known that SRBC-DTH in mice has an optimum sensitizing dose of SRBC. Induction of suppressor T cells has been considered as a mechanism for the reduced response by an over-sensitizing dose of SRBC. It is widely accepted that treatment with cyclophosphamide before antigen increases the immune response against SRBC by eliminating the precursors of suppressor T cells[27,28], which also occures in the case of tumor immunity[29~32]. In the present results, the pretreatment with cyclophosphamide did not affect the response in mice sensitized with lower doses (10^5-10^6) of SRBC and enhanced it in mice sensitized with higher doses (10^7-5×10^8). Therefore, effects of morphine on 5×10^5 SRBC-DTH and/or 10^8 SRBC-DTH were examinded (Fig. 5). Morphine showed a tendency to suppress 5×10^5 SRBC-DTH and suppressed 10^8 SRBC-DTH, while morphine reduced the thymus weight. These results suggest less participation of suppressor T cells in the response of 10^8 SRBC-DTH. Naloxone interrupted the immunosuppressive effect of morphine. Because morphine can diminish immune functions, the host immune response to tumor cells may be diminished, but the apecific effector cell or cells to be involved in vivo have yet to be

determined.

Two possible mechanisms may be suggested to explain the reduction of SRBC-DTH and accompanying atrophy of the spleen and thymus that were observed with consecutive administration of morphine. Cold stress has long been known to suppress immune function ; even mild reductions in environmental temperature can reduce lymphocyte-mediated immunity[33]. The hypothermia often observed in opiate-treated animals therfore might act as a "cold stress" to suppress immunity. In the present study, consecutive administration of morphin-treated mice did demonstrate a significant reduction of body temperature (date not shown). These data argue against morphine-induced hypothermia as being responsible for the immunomodulatory effects.

Another possible explanation for morphine-induced immune depression could be the indirect effect of excessive cholinergic stimulations. Morphine is a potent acetylcholine stimulant. Since cholinergic receptors have been shown to be present on lymphocytes and monocytes[34,35], it is reasonable to expect acetylcholine to act directly on lymphoid and accessory cells. Although in vitro studies show that cholinergic agents appear to exert a predominantly immunopotentiating effect[34], a direct correlation between organophosphate-induced cholinergic stimulation and immunosuppression in vivo has been established[36].

An alternative mechanism is that the suppression of these immunologic endpoints with consecutive administration of morphine may be secondary to the glucocorticoid mobilizing effect of morphine[1,37]. Indeed, glucocorticoids are known to inhibit immune function[38], and the immunosuppressive effect of chronic ethanol administration is partially mediated through the release of glucocorticoids[38].

Findings such as these, as well as other effects in the spectrum of biologic responses to morphine, have prompted us to suggest that consecutive administration of morphine in itself represents a "stressful" stimulus to the organism. For example, it is interesting that many of the effects observed in stressed animals such as analgesia, hypercholesterolemia, hyperphagia and changes in thermoregulation and a variety of endocrine parameters are also observed in morphine-treated animals[1]. Morphine-induced immunomodulation, therfore, represents another effect of the narcotic shared with chronic stress, as stressful stimuli are known to have marked effects on immune function[1,39].

Direct enhancement of tumor growth is another possible means of enhancing tumor growth. But, the growth-inhibiting properties of opiates in cultured tissues are well known and tumor cells grown retardation in the presence of opiates and endorphine that can be blocked by coadministration of naloxone[40]. Moreover, in the present study morphine inhibited tumor cell in cultures. Therefore, direct action of morphine is not likely to play an important role.

Our results indicate that morphine can remarkably alter the coure of experimental tumor cells. Our evidence suggestes that opiate receptors and endogenous opioid peptides play a role in tumor growth. The underlying explanation for ability of morphine to alter EL-4 and P388 lymphoma growth in mice is not readily apparent. Further studies are needed to clarify the mechanism of development of EL-4 and P388 lymphoma growth induced by morphine.

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