Antinociception Following Implantation of AtT-20 and Genetically Modified AtT-20/hENK Cells in Rat Spinal Cord

Hope H. Wu[1], Steven C. McLoon[2] and George L. Wilcox[3]

[1,3]*Department of Pharmacology,* [2,3]*Graduate Program in Neuroscience and* [2]*Department of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis, MN 55455, USA*

SUMMARY

AtT-20 cells, which produce β-endorphin, and AtT-20/hENK cells, which are AtT-20 cells transfected with a proenkephalin gene, were implanted in the rat spinal subarachnoid space in an effort to produce an antinociceptive effect. Host rats were tested for antinociceptive activity by standard nociceptive tests, tail flick and hot plate. Although cell implants had minimal effect on the basal response to thermal nociceptive stimuli, administration of the β_2-adrenergic agonist isoproterenol produced antinociception in the cell-implanted group but not in the control group. The antinociceptive effect of isoproterenol was dose-related and could be blocked by the opioid antagonist naloxone. Immunohistochemical analysis of spinal cords revealed the presence of enkephalin-negative cells surrounding the spinal cord of rats receiving AtT-20 cell implants, and enkephalin-positive cells surrounding the spinal cord of rats receiving AtT-20/hENK cell implants. These results suggest that opioid-releasing cells implanted around rat spinal cord can produce antinociception and may provide an alternative therapy for chronic pain.

KEY WORDS

antinociception, AtT-20 cell implantation, beta-endorphin, beta-adrenoceptors, enkephalin, spinal cord

INTRODUCTION

Analgesics remain under-administered in chronic and terminal cancer patients /5/. Although recently developed procedures for continuous infusion of opioids at the spinal level provide powerful analgesic effects, the infusion may fail when problems occur related to the infusion catheter, such as infection, occlusion or catheter dislocation /10,13,23,25/. Therefore, alternative ways to control pain are desirable. One approach may be to implant tissues or cells that secrete analgesic substances around the spinal cord to reduce the spinal transmission of pain. Spinal implantation of adrenal medullary chromaffin cells, cells known to release opioids and catecholamines, resulted in measurable antinociception in rats /32,33/, in decreased indications of pain in a rat model /3/ of neuropathic pain /12/ and in pain relief in terminal cancer patients /34/. In view of the difficulty associated with obtaining adrenal cells, an alternative source of opioid-secreting cells for transplantation would be useful. In the present study, two mouse cell lines, AtT-20 and AtT-20/hENK, were tested for antinociceptive properties when implanted adjacent to the spinal cord in the rat.

AtT-20 cells were originally derived from a mouse anterior pituitary tumor /6/. These cells synthesize and secrete the opioid peptide β-endorphin /15,20,29/. β-Endorphin, when administered intrathecally, produced antinociception in rats /11,45,46/ and analgesia in humans

Reprint address:
Dr. George L. Wilcox
Department of Pharmacology
University of Minnesota
3-249 Millard Hall
435 Delaware Street SE
Minneapolis, MN 55455, USA

/24,40/. The genetically modified AtT-20/hENK cell line was derived from the AtT-20 line by introduction of a plasmid containing the human proenkephalin gene /8/. These cells express proenkephalin protein which is cleaved to form free enkephalins. Enkephalins, when administered intrathecally, have also been shown to produce antinociception in rat /45,47/ and mouse /17/. It was expected that the β-endorphin released from AtT-20 cells or the β-endorphin and enkephalin released from AtT-20/hENK cells following implantation adjacent to the spinal cord would result in reduced pain sensitivity in host animals.

Release of opioids from these cells can be enhanced by certain drugs in culture. β-Endorphin release from AtT-20 cells was increased by corticotropin releasing factor (CRF) stimulation /1,2/. This action was suggested to involve increased production of cAMP /2/. Therefore, intrathecally administered CRF should provide a way to enhance antinociceptive effects of the implanted cells by increasing β-endorphin release. However, the utility of CRF for these studies is confounded by the recent finding that CRF has spinal antinociceptive activity of its own through κ opioid receptors in the writhing test /38/ and antagonizes morphine antinociception in the tail flick test /37/; therefore, an alternative method was sought to promote opioid secretion from these cells. AtT-20 cells possess β_2-adrenoceptors coupled to a stimulatory G-protein, which, when activated, increases cAMP production /28/. The β-adrenergic agonist isoproterenol stimulated ACTH release from AtT-20 cells /2,28/. Since CRF and isoproterenol stimulate cAMP formation and ACTH release through parallel mechanisms /19,27/, it was predicted that isoproterenol, like CRF, would also stimulate β-endorphin release from AtT-20 cell implants. Unlike CRF, isoproterenol has been reported to have no antinociceptive activity by itself /22,48/; an antinociceptive effect induced by isoproterenol would most likely result from stimulation of opioid secretion from the implanted cells. Therefore, isoproterenol administered intrathecally was used as a stimulator of opioid secretion in the present study.

The results of this study showed that intrathecally implanted AtT-20 cells had an antinociceptive effect when stimulated by isoproterenol. Isoproterenol-induced antinociception in rats implanted with AtT-20 cells was blocked by the opioid antagonist naloxone, suggesting that β-endorphin secretion from the implants accounted for this activity. Isoproterenol also produced antinociception in rats implanted with AtT-20/hENK cells. Preliminary results have been reported previously in abstract form /44/.

MATERIALS AND METHODS

Cell culture and preparation for implantation

AtT-20 cells and AtT-20/hENK cells (a gift from Dr. M. Martin) were grown in Ham's F-10 medium containing 12% horse serum and 3% fetal calf serum under 10% CO_2 and 90% air at 37°C. The cells were removed from the culture flasks, centrifuged at 500 *g* for 5 minutes, resuspended in PBS (10 mM phosphate / 0.9% NaCl, pH 7.4) solution and counted using a hemocytometer. The cells were again centrifuged and resuspended in PBS at various concentrations for intrathecal (i.t.) administration to recipient animals.

Animals and cell implantation

Recipients were male Sprague-Dawley rats weighing 75-100 g (Harlan Sprague-Dawley, Madison, WI). Small rats (one month old) were used to facilitate i.t. injection by direct lumbar puncture. Animals were maintained on a 12-hour light/dark cycle with chow and water available *ad libitum*. Ten μl cell suspensions containing 10^6 cells were injected into the lumbar subarachnoid space according to the direct lumbar puncture technique described by Hylden and Wilcox /16/ and as modified by Wilcox /41/. The number of cells used per animal was determined by the maximum number of cells that could be easily suspended in 10 μl of medium and injected though a 27 ga needle. The effect of different volumes of cell suspension was not examined in the present study. In the experiments using isoproterenol stimulation, 10 μl of cell suspensions containing 4

x 10^5 cells were used. All i.t. injections in the present experiments were via 1 cm 27 ga needles. Control animals received 10 µl PBS i.t. During the three week duration of an experiment, the motor ability of animals was observed daily, and the animals were weighed every other day during the first week and weekly thereafter.

Antinociceptive testing

Nociceptive sensitivity was measured before and 1, 3 and 5 days after cell implantation. Two standard nociceptive tests, tail flick and hot plate, were used sequentially. For the tail flick test, a radiant heat source was applied to the tail /9/, and the time required for the animal to remove its tail from the heat source was measured. The baseline response time in the absence of treatment was 3.0 ± 0.1 seconds, and the cut-off time was set at 10 seconds. In the hot plate test the temperature of an aluminum plate (20 x 35 cm) in a clear plastic enclosure (34 cm high) was maintained at 52°C (± 0.5°C) by circulating hot water /42/. Each subject was placed on the hot plate and the latency to jump or lick its hind paw was measured. The baseline response time in the hot plate test was 24 ± 2 seconds and the cut-off time was set at 60 seconds. These maximum cut-off latencies were set to avoid tissue damage and were determined to be more than three standard deviations above the control mean for several pooled groups of control animals. The percent maximum possible effect (MPE) was determined in the usual way [% MPE = (postdrug latency - predrug latency)/(cut-off - predrug latency) x 100%].

Drugs and procedures for antinociceptive testing

Isoproterenol (Aldrich Chemical Company, Inc., Milwaukee, WI) was prepared in physiological saline containing the cAMP phosphodiesterase inhibitor ZK 62711 (a gift from Dr. P. Y. Law). The ZK 62711 was dissolved in absolute ethanol (30 mg/ml) then diluted with physiological saline to a final ethanol concentration of 1.0%. Naloxone hydrochloride (E.I. Dupont Co., Garden City, NJ) was prepared in physiological saline (0.3 mg/ml). Two to 9 days after cell implantation, the drugs were given by i.t. injection. All i.t. injections were made in a volume of 10 µl. Nociceptive sensitivity was measured before and after drug application. The drug experiments using i.t. injection were limited to 9 days after cell implantation because the increased size of the rats after this point made intrathecal drug injections more difficult. We were therefore unable to follow the antinociceptive effects of the implants at later times.

Immunohistochemistry

Three days after cell implantation, 2 rats of each cell-implanted group were anesthetized with ether and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The spinal cords were removed and post fixed in 4% paraformaldehyde for 2 hours at 4°C, then placed in 20% sucrose / 0.1 M phosphate buffer overnight at 4°C. The spinal cords were embedded in tragacanth gum and sectioned on a cryostat at 12 µm. Sections were incubated in rabbit anti-met-enkephalin antisera (a gift from Dr. R. P. Elde) at 1:100 in PBS /21/ followed after rinsing by incubation in goat-anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate (Jackson Immunoresearch Laboratories, Inc.) at 1:500 in PBS. The sections were examined and photographed with a microscope under epifluorescence illumination.

Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) followed by the Fisher PLSD test for multiple post-hoc comparisons among groups. Differences were considered to be significant if $p<0.05$.

RESULTS

Nociceptive thermal responses after AtT-20 and AtT-20/hENK cell implants

Three groups of 22 rats each received an i.t. injection of AtT-20 cells, AtT-20/hENK cells or PBS. The rats were tested in the tail flick and hot plate tests 1 day before and 1, 3 and 5 days after implantation. Pre- and post-cell implantation, the tail flick and hot plate response latencies to

nociceptive stimuli were compared. In the tail flick test, the degree of antinociception (% MPE) of the group receiving AtT-20/hENK cell-implants was higher than that of the AtT-20 cell-implanted group and the control group (Fig. 1A). There was no evidence for antinociception with any of the three groups in the hot plate test (Fig. 1B).

Effects of isoproterenol

Because basal opioid release from the implanted cells appeared inadequate to produce substantial antinociception, we sought to increase the opioid secretion from the cell implants by stimulation with the β-adrenergic agonist isoproterenol. Three days after implantation of AtT-20 cells, isoproterenol was coadministered with 10 μg ZK 62711, a phosphodiesterase inhibitor /35/. ZK 62711 was used to reduce enzymatic destruction of cAMP and to prolong the effect of isoproterenol /4,36/. This dose of ZK 62711 alone affected neither the tail flick nor the hot plate latencies post drug injection (Fig. 2). In the tail flick test the antinociceptive effect of isoproterenol (40 nmol, i.t.) in rats receiving AtT-20 implants was significantly higher than control groups at 3 and 10 minutes (Fig. 2). At 3 minutes in the hot plate test, both the AtT-20 and control groups showed an antinociceptive effect; the effect in the control group was considered to be due to the stress of handling during the injection. At 10 minutes in the hot plate test, partial antinociception was maintained in the AtT-20 cell-implanted group but not in the control group.

Nine days after AtT-20 cell implantation, tail flick and hot plate response latencies were determined before and 5 minutes after isoproterenol injection. Before isoproterenol, tail flick and hot plate response latencies showed no difference between AtT-20 cell-implanted and control groups. However, isoproterenol (12, 40 and 120 nmol i.t. coadministered with ZK 62711) produced dose-related tail flick (Fig. 3A) and hot plate (Fig. 3B) antinociception in the AtT-20 cell-implanted group. The highest dose of isoproterenol (120 nmol) did not produce antinociception in the control group. The anticiceptive effect of isoproterenol in the AtT-20 cell-implanted group could be blocked by coadministration of the opioid antagonist naloxone (3μg, i.t.), a dose that had no effect on its own in any of the three groups.

Isoproterenol also produced antinociception in rats receiving AtT-20/hENK cell implants, although these cells had lower apparent efficacy than AtT-20 cells. Figure 4 presents the results for eight individual rats 2 days after receiving AtT-20/hENK cells. Four rats showed higher tail flick response latency at 5 minutes after receiving isoproterenol (12 nmol, i.t.) than that of the control group. The antinociceptive effect of 12 nmol isoproterenol was smaller with AtT-20/hENK cell implants (19±5% MPE) than that observed with AtT-20 cell implanted rats (41±14% MPE).

Rat hosts

Intrathecal implantation of AtT-20 cells was not lethal to host rats. The size and the motor ability of 7 rats receiving 10^6 AtT-20 cells did not differ from control rats for as long as six months following cell implantation (data not shown). The growth rate of the rats, however, was decreased in the AtT-20/hENK cell-implanted group (Fig. 5). Also, seven days after AtT-20/hENK cell implantation, 43% of host rats manifested hind limb paralysis, indicating that AtT-20/hENK cells may differ from AtT-20 cells in their metastatic potential.

Immunohistochemistry

Three days after cell implantation, the spinal cords of rats from the three groups were examined histologically. Sections of spinal cords processed for enkephalin immunoreactivity revealed the presence of enkephalin-positive cells around the spinal cord of an AtT-20/hENK cell-implanted rat (Fig. 6A,C), and the presence of enkephalin-negative cells around the spinal cord of an AtT-20 cell-implanted rat (Fig. 6B,D). No cells were seen around the spinal cord of control rats.

DISCUSSION

The present study showed that intrathecal implantation of AtT-20 or genetically modified AtT-20/hENK cells had an antinociceptive effect apparently mediated by opioid receptors. This effect was revealed when the cell implants were

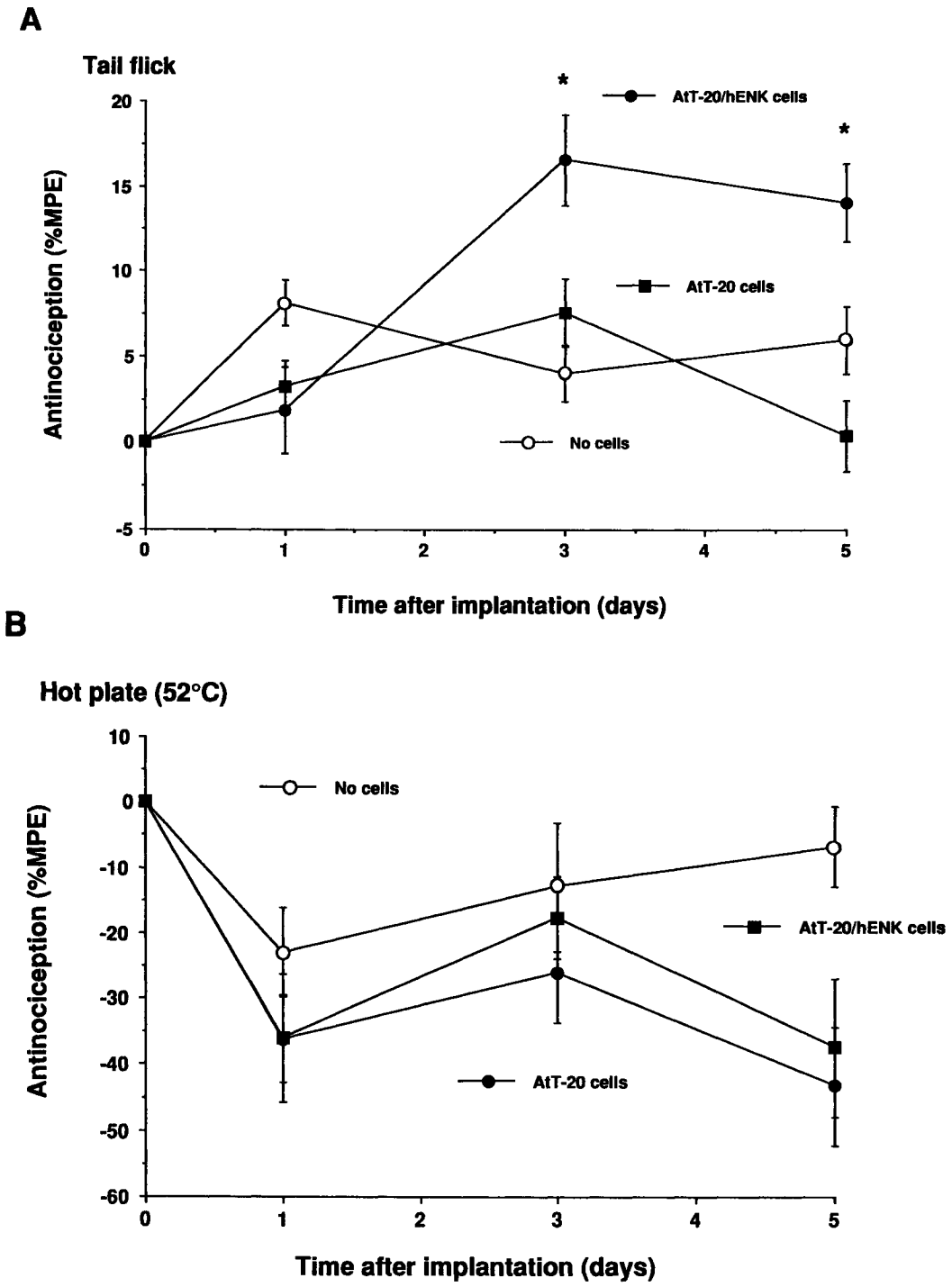

Fig. 1: The percent maximum possible effect (%MPE) of base line tail flick (A) and hot plate (B) latencies in rats after receiving 10^6 AtT-20 cells, 10^6 AtT-20/hENK cells or no cells i.t. In the tail flick test, a two-factor, repeated measures ANOVA showed significant differences among the three groups ($F_{2,63}=5$, $p<0.01$). The AtT-20/hENK cell-implanted group showed more antinociception than the AtT-20 cell-implanted and control groups. Significant post-hoc comparisons are indicated by *($p<0.05$). In the hot plate test, a two-factor, repeated measures ANOVA showed no significant differences among the three groups ($F_{2,63}=1.8$, $p=0.12$). Each point represents the mean ± SEM (22 rats in each group).

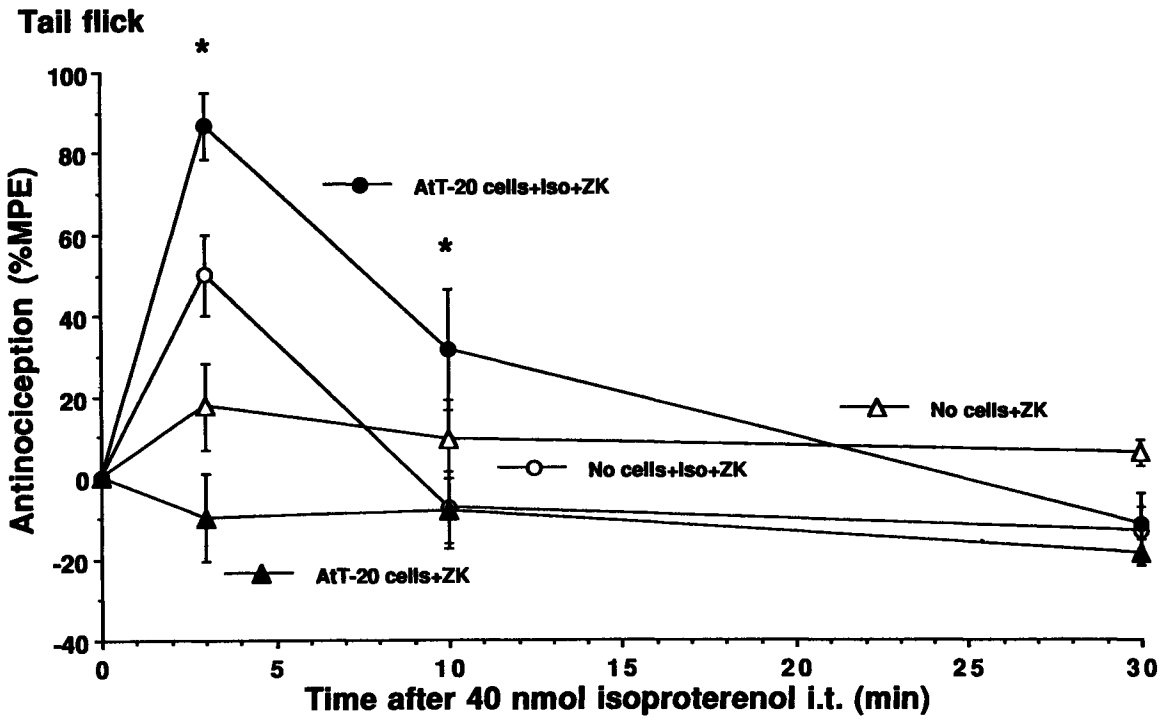

Fig. 2: The β-adrenergic agonist isoproterenol (40 nmol, i.t.) together with the cAMP phosphodiesterase inhibitor ZK 62711 (10 μg, i.t.) in rats 3 days after receiving 4 x 10^5 AtT-20 cells i.t. produces antinociception in the tail flick test. A three-factor repeated measures ANOVA indicated that the antinociceptive effect of isoproterenol was statistically significant ($F_{1,24}$=16, p<0.001). Significant post-hoc comparisons are indicated by * (p<0.05). The AtT-20 cell-implanted group showed more antinociception than the control group. Each point represents the mean ± SEM (7 rats in each group).

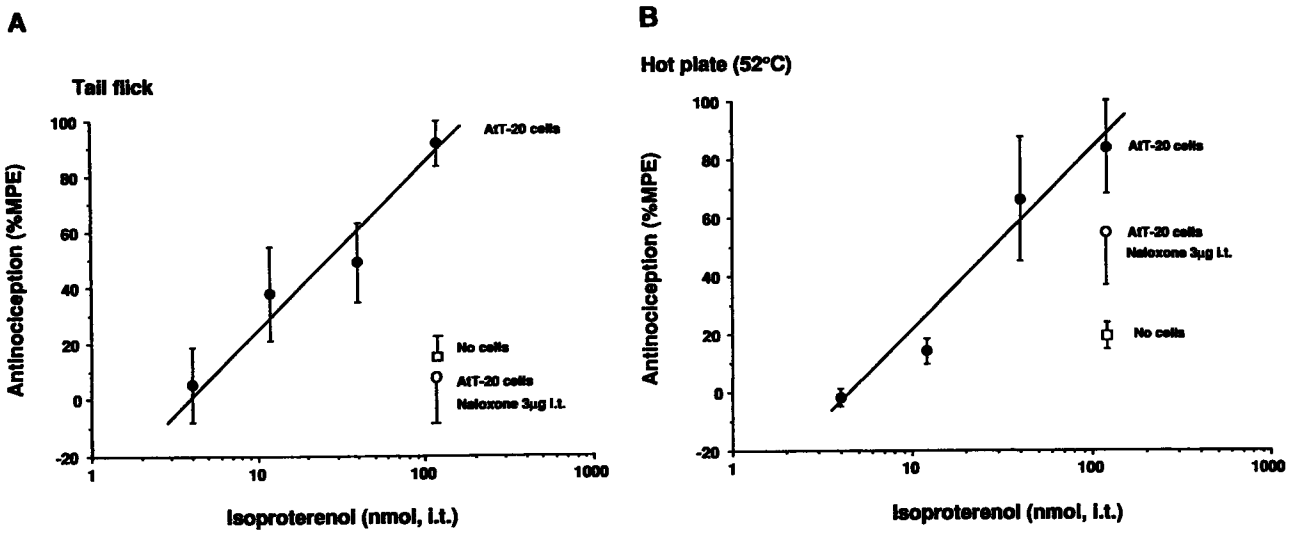

Fig. 3: Dose-related, isoproterenol-induced antinociception (12, 40 or 120 nmol isoproterenol) together with the cAMP phosphodiesterase inhibitor ZK 62711 (10 μg, i.t.) in rats 9 days after receiving 4 x 10^5 AtT-20 cells i.t. was blocked by the opioid antagonist naloxone (3 μg, i.t.) in the tail flick (A) and hot plate (B) tests. Each point represents the mean ± SEM (5 rats in each group).

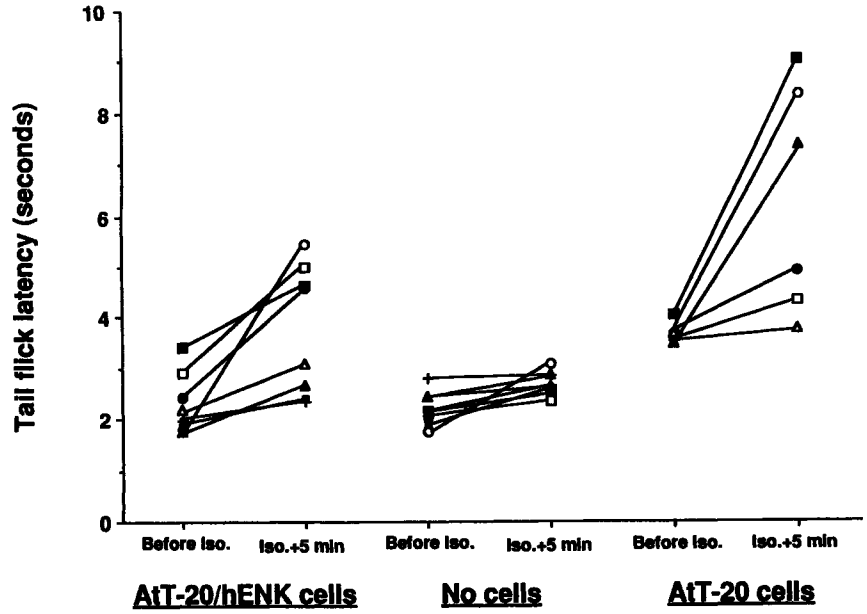

Fig. 4: The β-adrenergic agonist isoproterenol (12 nmol, i.t.) together with the cAMP phosphodiesterase inhibitor ZK 62711 (10 μg, i.t.) produced antinociception in the tail flick test in rats 2 days after receiving 10^6 AtT-20/hENK or 3 days after receiving 4 x 10^5 AtT-20 cells i.t. A one-factor ANOVA indicated a statistically significant ($F_{2,19}$=5, $p<0.05$) antinociceptive effect of isoproterenol in the AtT-20/hENK cell-implanted group compared with the control group. There was no significant difference between the AtT-20/hENK and the AtT-20 group (each line represents one rat).

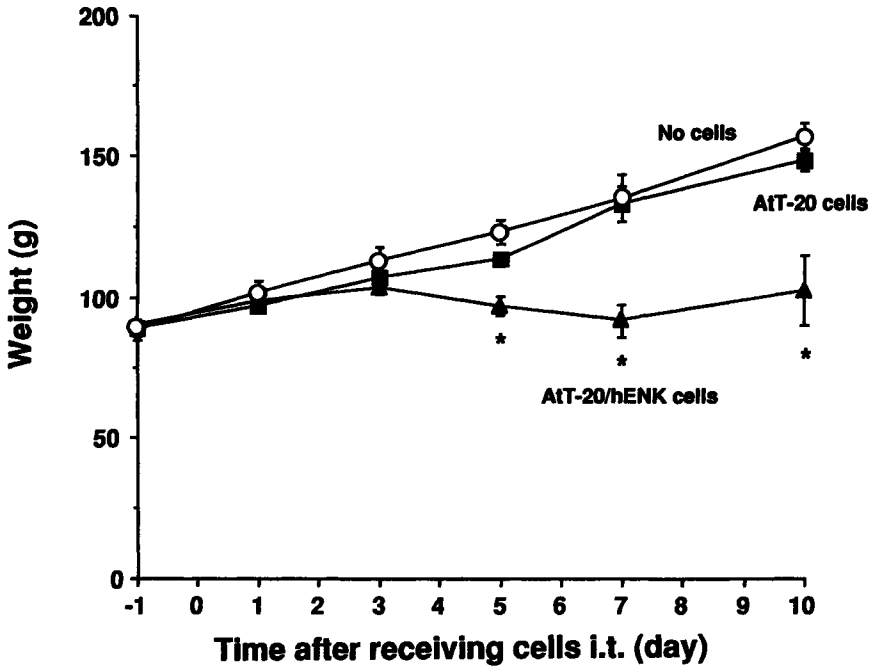

Fig. 5: The body weight of rats receiving 10^6 AtT-20 cells or 10^6 AtT-20/hENK cells i.t. The weight gain of rats receiving AtT-20 cells did not differ from control. The AtT-20/hENK cell-implanted group had lower weight gain than the AtT-20 cell-implanted or control groups during 10 days after cell implantation (two-factor, repeated measures ANOVA, $F_{2,18}$=9, $p<0.005$). Significant post-hoc comparisons are indicated by *($p<0.05$). Each point represents the mean ± SEM (7 rats in each group).

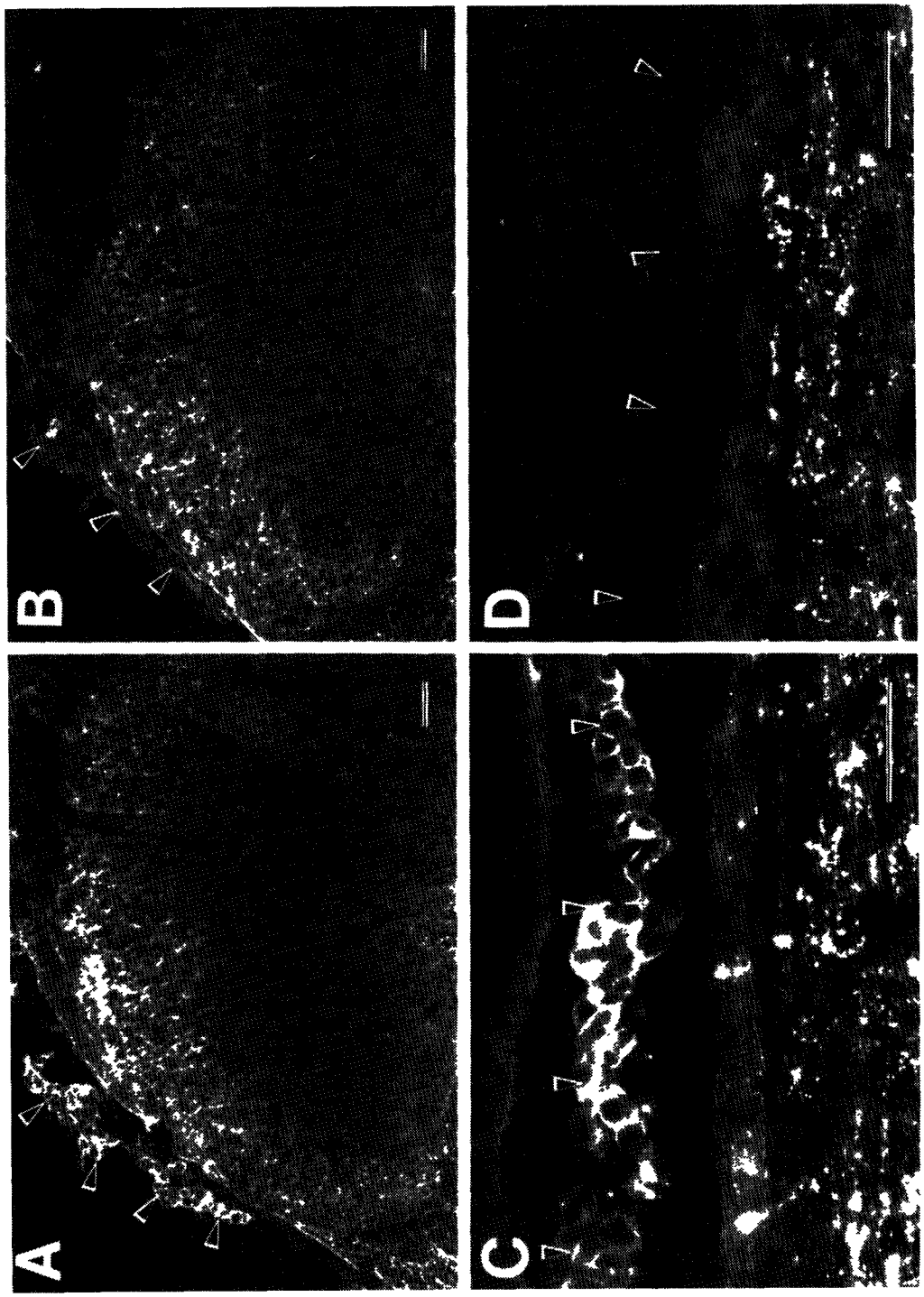

Fig. 6: Photomicrograph of rat spinal cord longitudinal sections with implanted cells (arrowheads). The sections were processed for met-enkephalin immunohistochemistry. Immunofluorescent staining is apparent within the implanted AtT-20/hENK cells (A and C) but not in AtT-20 cells (B and D). Bar indicates 50 μm.

pharmacologically stimulated to secrete opioids by administration of isoproterenol.

AtT-20/hENK cell implants produced a partial antinociceptive effect in the tail flick test. AtT-20 cell implants did not affect the baseline tail flick response latency, and neither AtT-20 nor AtT-20/hENK cell implants affected the baseline hot plate response latency. This result agrees with observations made with adrenal medullary chromaffin cell implants /32/. This lack of antinociception in the tail flick and hot plate tests does not completely discount the analgesic utility of such cell implants. Despite failure of adrenal medullary chromaffin cell implants in rat spinal cord to alter the baseline tail flick and hot plate latencies, implants in rats with neuropathic pain /12/ and humans with terminal cancer pain /34/ were effective.

The effect of implant-induced antinociception in the absence of cell stimulation was minimal with AtT-20 cell implants and small but significant with AtT-20/hENK cell implants in the tail flick test. This may be due to a low spontaneous secretion of opioid by these cells /8,15/. It was reasoned that antinociception might be revealed by use of a pharmacological agent to stimulate opioid secretion from the implants. A similar observation was reported with adrenal medullary chromaffin cell implants, which produce antinociception only after nicotine stimulation of release of opioid peptides and catecholamines from the implants /30-32/. Our results showed that isoproterenol produced antinociception in rats implanted with AtT-20 or AtT-20/hENK cells. Since isoproterenol is able to stimulate release from AtT-20 cells *in vitro* /2,28/, the antinociceptive effects of isoproterenol in rats receiving AtT-20 cell implants probably resulted from β-endorphin secretion from the implants. The observation that isoproterenol-induced antinociception in the AtT-20 cell-implanted rats was completely blocked in the tail flick test and partially blocked in the hot plate test by the opioid antagonist naloxone supports this contention.

Our results indicate that antinociception produced by isoproterenol lasts only about 10 minutes. Stress-induced analgesia cannot account for all the antinociception observed because control groups, which received the same injections, showed little antinociception and because the effects of these cell stimulators were dose-related. The short duration of antinociception may result from other causes. It has been shown that the duration of antinociception induced by spinal administration of β-endorphin is dose-dependent: 1 nmol β-endorphin i.t. lasts for 1 hour and 10 nmol β-endorphin i.t. lasts for 2 hours, while 0.1 nmol β-endorphin i.t. is inactive /11/. The amount of β-endorphin secreted from the implants may be quite small, yielding only a short duration of action. In addition, we have observed that mice receiving AtT-20 cell implants developed tolerance to opioids (i.t.) /43/. Rats implanted with AtT-20 cells may also develop tolerance to β-endorphin, reducing the response to β-endorphin released from the cell implants by isoproterenol stimulation. Furthermore, β-adrenergic receptor desensitization /14,36/ may contribute to the short duration of isoproterenol antinociception.

Contrary to Yaksh's observation /22,48/, the present results indicate that isoproterenol induced a small antinociceptive effect in control rats. Differences in the i.t. injection method between our laboratory and Yaksh's laboratory may account for this difference. Yaksh's group used chronic indwelling intrathecal catheters, while the present study used direct lumbar puncture to deliver isoproterenol. The small antinociceptive effect observed in the present study may result from the stress of handling during the drug injection.

AtT-20/hENK cell implants secrete enkephalin in addition to β-endorphin. Since both enkephalin and β-endorphin produced antinociception when administered to the spinal cord /45-47/, it was expected that AtT-20/hENK cell implants might result in a greater antinociceptive effect than that detected with AtT-20 cell implants. The present study showed that unstimulated AtT-20/hENK cells produced more antinociception in the tail flick test than that produced by AtT-20 cell implants. By contrast, isoproterenol stimulation produced more antinociception with AtT-20 cells than with AtT-20/hENK cells. It has been reported that met-enkephalin antagonizes while leu-enkephalin potentiates morphine antinociception /7,18,26,39/. The proenkephalin gene transfected in genetically modified AtT-20/hENK cells contains six met-

enkephalin sequences and one leu-enkephalin sequence /8/. The preponderance of met-enkephalin in AtT-20/hENK cells might modulate β-endorphin antinociception negatively, reducing antinociception in AtT-20/hENK cell-implanted rats.

The present study showed that rat hosts survived indefinitely with intrathecal implantation of AtT-20 cells but not with AtT-20/hENK cells. Preliminary studies showed that when AtT-20 and AtT-20/hENK cell-implanted rats were treated daily with the immunosuppressant cyclosporin A (1 mg/kg, i.p.) after implantation, both groups of rats lost weight and developed hind limb paralysis within 7 days. It was observed that the growth of AtT-20 and AtT-20/hENK cell lines in culture exhibited different characteristics. The AtT-20 cells floated in culture media while most of the AtT-20/hENK cells adhered to the bottom of the culture flasks. The different characteristics exhibited by AtT-20 and AtT-20/hENK cells in culture may reflect differences in the metastatic potential of the two cell lines. Interestingly, mice injected with fewer cells (10^5) remained healthy for at least 1 month /43/.

Histological studies suggested that both AtT-20 and AtT-20/hENK cells survived implantation. Immunohistochemistry showed the presence of enkephalin-positive cells surrounding the spinal cords of AtT-20/hENK cell-implanted rats. Cells, which were not present in control animals, surrounded the spinal cords of AtT-20 cell-implanted rats, but these were enkephalin-negative. This result was as expected, because only the genetically modified AtT-20/hENK cell line secretes enkephalin /8/.

This study has identified and characterized pharmacologically an antinociceptive effect of opioid-producing AtT-20 and genetically modified AtT-20/hENK cells implanted around rat spinal cord. Implantation of cell lines may provide a method to control chronic pain in patients. The present study is an initial step in the development of cell lines for transplantation in pain syndromes. In future studies, it will be important to determine whether the implanted cells release opioid peptides for long periods post implantation, and whether encapsulation of the implanted cells would reduce the metastatic potential of the cells without compromising their antinociceptive effect.

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