Sensation of Abdominal Pain Induced by Peritoneal Carcinomatosis Is Accompanied by Changes in the Expression of Substance P and μ-Opioid Receptors in the Spinal Cord of Mice

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ABSTRACT

Background: Patients with peritoneal carcinomatosis often report abdominal pain, which is relatively refractory to morphine. It has been considered that a new animal model is required to investigate the mechanism of abdominal pain for the development of optimal treatments for this type of pain.

Methods: To prepare a peritoneal carcinomatosis model, highly peritoneal-seeding gastric cancer cells, 60As6, were implanted into the abdominal cavity. The nociceptive modality for pain-related behavior was assessed in terms of withdrawal behavior in response to mechanical stimuli and hunching behavior. Tissue samples from mouse dorsal root ganglia and spinal cord were subject to immunohistochemistry and real-time reverse transcription polymerase chain reaction.

Results: Mice with peritoneal dissemination showed significant hypersensitivity of the abdomen to mechanical stimulation and spontaneous visceral pain-related behavior. There was a significant increase in c-Fos–positive cells in the spinal cord in tumor-bearing mice. Those mice exhibited a remarkable increase in substance P-positive neurons in the dorsal root ganglia (control *vs.* tumor, 15.4 ± 1.1 *vs.* 24.2 ± 3.6, $P < 0.05$, n = 3). A significant decreases in μ-opioid receptor expression mainly in substance P-positive neurons was observed in tumor-bearing mice (69.3 ± 4.9 *vs.* 38.7 ± 0.9, $P < 0.05$, n = 3), and a relatively higher dose of morphine was required to significantly reverse the abdominal hypersensitivity.

What We Already Know about This Topic

- Some patients with cancer have tumor spread throughout the abdominal peritoneum
- The disease of widespread peritoneal tumors, carcinomatosis, can be painful and refractory to conventional analgesic therapies

What This Article Tells Us That Is New

- The investigators developed a mouse model of carcinomatosis
- Behavioral studies indicate pain-related responses are present, and as in patients, the responses are resistant to morphine
- In the future, experimental pain therapies can be studied

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Conclusion: Both the up-regulation of substance P and down-regulation of μ-opioid receptor seen in the dorsal root ganglia may be, at least in part, responsible for the abdominal pain-like state associated with peritoneal carcinomatosis.

THE moderate or severe pain associated with cancer can impair dramatically the quality of life and the survival of patients and affects 64% of those with metastatic or advanced-stage cancer.[1] The perceived intensity of this pain depends on the specific type of cancer and its location, as well as the each patient's sensitivity to pain.[2] Pain associated with cancer generally is treated with opioids, nonsteroidal antiinflammatory drugs, corticosteroids, local anesthetics, antidepressants, and anticonvulsants, either alone or in combination.[3,4] Despite the availability of these various medicinal treatments, it can be difficult to control pain in some patients with terminal cancer. Accordingly, more effective treatments are needed. Our poor understanding of the mechanism of pain associated with cancers continues to stand as a major obstacle to the discovery of novel analgesics to address this need.

Peritoneal carcinomatosis has been defined as the complex sequence of events by which tumor cells disseminate from their primary organ of origin to establish independent metastatic deposits on the visceral and parietal peritoneal lining of the abdominal cavity. Advanced gastric cancer with peritoneal dissemination is one of the most difficult forms of gastric cancer to treat, and its prognosis remains poor.[5] Patients with peritoneal carcinomatosis report constant, aching abdominal pain. Characteristically, it is poorly localized and is worsened by pressure on the abdomen. Although there have been no rigorous clinical studies, most experts agree that opiate analgesics are relatively ineffective for the treatment of abdominal pain caused by cancerous peritonitis. Compared with somatic pain, which is easily localized and characterized by distinct sensations, visceral pain is diffuse and poorly localized, typically referred to somatic sites, and associated with stronger emotional and autonomic reactions.[6] The abdominal viscera receive dual extrinsic innervation (*e.g.*, spinal and vagal afferents), and accumulating evidence has revealed that there are significant differences in the functions of different nerves innervating the same organ.[6] Adequate stimuli for the production of visceral pain include the distension of hollow organs, traction on the mesentery, ischemia, and endogenous chemicals typically associated with inflammatory processes.[6] Thus, visceral pain differs from somatic pain in several important ways. Several new animal models have been developed for the investigation of cancer pain.[7] The first animal models were developed for the study of primary and metastatic bone tumors,[8] and these were followed by nonbone models of cancer pain that resemble other malignant lesions.[9,10] These animal models were established to enhance our understanding of the neurobiology, pharmacology, and molecular mechanisms of tumor pain.[11–14] However, these are mostly models for somatic pain. The development of optimal analgesic medications for the treatment of abdominal pain caused by cancerous peritonitis has been hindered by our incomplete understanding of the underlying mechanisms, mainly because of a lack of appropriate animal models to study. In this study, we used 60As6 gastric cancer cells, which are a highly peritoneal-seeding cell line, to develop a novel mouse model of abdominal pain caused by cancer-related peritonitis. Information obtained with this model has provided new insight into the mechanisms that underlie pain caused by cancerous peritonitis and may aid the establishment of potential mechanism-based therapies for treating this pain state.

Materials and Methods

All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain[15] and were approved by the Committee for Ethics of Animal Experimentation of National Cancer Center (Tsukiji, Tokyo, Japan). In the experiments, efforts were made to minimize the numbers of animals used and their suffering.

Animals

Male C.B17/Icr-scid mice and Institute of Cancer Research mice weighing 22–25 g were used. Mice were purchased from CLEA Japan (Tokyo, Japan) and housed at a room temperature of 23 ± 1°C with a 12-h light–dark cycle. The mice were maintained under specific pathogen-free conditions and provided sterile food, water, and cages.

Cell Lines and Culture

A human scirrhous gastric cancer cell line, HSC60, was established as described previously.[16] The highly peritoneal-seeding cell line, 60As6 was established from HSC60 by orthotopic tissue implantation into scid mice.[17] Briefly, a xenograft tumor of HSC60 cells was transplanted into the gastric wall of a scid mouse. For six iterations, we harvested ascitic tumor cells and performed the orthotopic inoculation of these cells into mice to establish a highly metastatic 60As6 cell line. This cell line was maintained in RPMI1640 medium supplemented with fetal calf serum (10%), 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate under an atmosphere of 5% carbon dioxide and 95% air at 37°C. To establish transfectants that expressed the luciferase gene, plasmid vectors carrying the firefly luciferase gene, which were called pLuc/Neo, and a transfection reagent, LipofectAMINE 2000 (Invitrogen, Carlsbad, CA), were used as recommended by the manufacturer. Geneticin (500 μg/ml; Invitrogen) was used to select stable transfectants, and transfected clones were screened for luciferase gene expression by detecting bioluminescence using an IVIS system (Xenogen, Alameda, CA). Clones that expressed the luciferase gene were referred to as 60As6Luc cells.

Intraperitoneal Inoculation of 60As6Luc Cells

The density of 60As6Luc cells was adjusted to 1×10^6 cells per 1 ml phosphate-buffered saline (PBS). In the experimental group, the cell suspension was injected into the abdominal cavity through a 26.5-gauge needle inserted into the central abdomen. In the control group, PBS was injected into the abdominal cavity instead of 60As6Luc cells.

Measurement of Tumor Growth Using Luciferase Imaging

For the measurement of tumor growth, whole-body luciferase imaging with an IVIS imaging system was used to visualize 60As6Luc cells under a 10-min integration time for image acquisition, as described previously.[18] Briefly, mice were injected intraperitoneally with 15 mg D-luciferin potassium salt in 1 ml PBS using a 26.5-gauge syringe. They were then kept anesthetized with isoflurane. The relative tumor metastasis burden was determined using Living Image software (version 2.50, Xenogen).

Acute Pancreatitis Model

Caerulein is an analog of cholecystokinin that leads to acute pancreatitis. Acute pancreatitis was induced by repeatedly injecting mice with caerulein (intraperitoneal administration; 50 μg/kg; six times at 1-h intervals).[19,20] Caerulein (Sigma Chemical Co., St. Louis, MO) was dissolved in physiologic saline.

Inflammatory Pain Model

With the mice anesthetized with isoflurane, the plantar surface of the right hind paw was injected with complete Freund's adjuvant (CFA; Mycobacterium tuberculosis; Sigma Chemical Co.) in a volume of 20 μl to create a model of persistent inflammatory pain.[21]

Behavioral Test

Hypersensitivity of the abdomen to mechanical stimulation was quantified by counting the number of withdrawal behaviors (withdrawal of the abdomen away from a von Frey filament, licking of the abdomen as a result of stimulation, or whole-body withdrawal) in response to the application of mechanical stimulation (von Frey filaments with a bending force of 0.02 g) to the abdomen.[19,20] Mice were placed on an increased wire mesh floor and confined under individual overturned black plastic boxes. The von Frey filaments were applied through the mesh floor to different points on the surface of the abdomen. Each filament was applied five times at intervals of 5–10 s, and again after a 1-min rest period for a total of 10 times. Nociceptive behavior was scored as follows: 0 = no response; 1 = immediate slight attempt to escape or light licking or scratching of the stimulated site; 2 = intense withdrawal of the abdomen or jumping. The reported values are the total scores for the responses to 10 challenges. The observer was unaware of the mouse's experimental condition. Behavioral testing was performed on days 14 and 28 after tumor inoculation.

Hunching behavior was examined as described previously with some modifications.[14] Briefly, mice were placed individually in the center of an open field arena and observed for 180 s. The hunching score was the total time (s) the mouse exhibited hunching behavior multiplied by the scoring factor, which was defined according to Sevcik *et al.*[14]: 0 = normal coat luster, displays exploratory behavior; 1 = mild rounded-back posture, displays slightly reduced exploratory behavior, normal coat luster; 2 = severe rounded-back posture, displays considerably reduced exploratory behavior, piloerection, intermittent abdominal contractions. Behavioral testing was performed on days 14 and 28 after tumor inoculation. The observer was unaware of the mouse's experimental condition. Behavioral testing was performed on days 14 and 28 after tumor inoculation.

In the model of CFA-injected inflammatory pain, hypersensitivity of the paw to mechanical stimulation was quantified by counting the number of paw withdrawals in response to the application of mechanical stimulation (von Frey filaments with a bending force of 0.02 g) to the right paw.[21] von Frey filaments were applied to the plantar surface of the hind paw for 3 s (two applications at an interval of at least 5 s). Paw-withdrawal behavior was scored as defined by Narita *et al.*[21]: 0 = no response, 1 = a slow and/or slight response to the stimulus, 2 = a quick withdrawal response away from the stimulus without flinching or licking, 3 = an intense withdrawal response away from the stimulus with brisk flinching and/or licking. The final score for each filament was the average of two scores per paw. Morphine hydrochloride (Sankyo, Tokyo, Japan) was dissolved in physiologic saline. Behavioral testing using morphine was performed at day 28 after tumor inoculation in tumor-bearing mice, at 6 h after the last injection of caerulein (50 μg/kg and six times) in the acute pancreatitis model, or at 3 days after the injection of CFA in the inflammatory pain model. In the current study, the analgesic effects of morphine or saline were assessed at 30 min (peak time) after subcutaneous injection by an observer who was blind to the type of treatment. The effects of morphine were assessed in terms of the percentage changes from the basal values (before morphine injection).

Immunohistochemistry

Twenty-eight days after the inoculation of tumor cells, mice were deeply anesthetized with isoflurane and perfused with paraformaldehyde (4%; pH 7.4). The spinal cord and dorsal root ganglia (DRG) were removed rapidly and postfixed in 4% paraformaldehyde for 2 h. Sections of the spinal cord (T10–T11) and DRG (T10–T12) were prepared as described previously.[22] Frozen sections of spinal cords and DRGs were cut at 20 and 10 μm, respectively, using a cryostat (Leica CM1510; Leica Microsystems, Heidelberg, Germany). The sections were blocked in normal horse serum (10%) in 0.01 M PBS for 1 h at room temperature. Each

primary antibody was diluted in 0.01 M PBS containing normal horse serum (10%; 1:5,000 c-Fos; Calbiochem, La Jolla, CA), 1:200 substance P (Neuromics, Edia, MN), 1:250 μ-opioid receptor (MOR; a gift from Masahiko Watanabe, M.D., Ph.D., Hokkaido University Graduate School of Medicine, Sapporo, Japan, October 2010), and anti-MOR antibody against the 1–38 amino acid sequence of the mouse MOR N-terminus[23] and incubated for 1 day at 4°C. They were then rinsed and incubated with each secondary antibody conjugated with Alexa 488 and Rhodamine Red for 2 h at room temperature. The slides were coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA), and the fluorescence of immunolabeling was observed by confocal microscopy (LSM510 Meta; Carl Zeiss, Jene, Germany). The anti-MOR antibody used in this study has been shown to be quite specific.[23] The dorsal horn was divided into a superficial layer (L1–2 laminae) and a deep layer (L3–5 laminae), and the number of c-Fos–positive cells was counted in each layer.[24] The cells in 10 randomly selected sections for each marker in each animal were counted, and the average value was determined for each animal. The number of MOR- and substance P-positive profiles in the DRGs was counted as described previously.[25] Briefly, the percentages of MOR- and substance P-positive profiles were determined by counting approximately 2,200 T10–T12 DRG neuronal profiles in tumor-bearing and control mice. We also determined the number of double-positive cells among 500–800 MOR-positive profiles in each mouse. Because we did not use a stereological approach, this quantification may have led to biased estimates of the true numbers of cells and neurons. All counting was performed by an assistant who was blind to the treatment group for the respective sections. To avoid counting neuronal cell bodies twice, for each DRG, we counted sections that were 50 μm apart.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the thoracic 6–13 th and L1–3 DRG using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. To obtain first-strand complementary DNA, 5 μg total RNA was incubated in 100 μl buffer containing 10 μM dithiothreitol, 2.5 mM $MgCl_2$, deoxyriboside-triphosphate mixture, 50 units of reverse transcriptase II (Invitrogen) and oligo(dT)12–18 (Invitrogen). Diluted complementary DNA (2 μl) was amplified in a rapid thermal cycler (LightCycler 480; Roche Diagnostics, Mannheim, Germany) in 10 μl LightCycler 480 SYBR Green I Master (Roche Diagnostics) and each oligonucleotide. Primer sequences for the genes of interest (substance P, calcitonin gene-related peptide [CGRP], MOR, and glyceraldehyde-3-phosphate dehydrogenase) are shown in table 1. Size and melting curve analyses were performed to confirm that polymerase chain reaction (PCR) amplicons were specific. To quantify the PCR products, LightCycler 480 quantification software was used to analyze the exponential phase of amplification and the melting curve, as recommended by the manufacturer. The amount of target messenger RNA (mRNA) in the experimental group relative to that in the control was determined from the resulting fluorescence and threshold values (C_T) using the 2-ΔΔCT method.[26] Each experiment was run twice, and samples were run in duplicate. For each sample, a ΔC_T value was obtained by subtracting the mean C_T value for the control gene (glyceraldehyde-3-phosphate dehydrogenase) from that for the gene of interest (substance P, CGRP, and MOR). The average ΔC_T value for the control group was then subtracted from the value of each animal in the experimental group to obtain $\Delta\Delta C_T$. The fold change relative to the control was then determined by calculating $2^{-\Delta\Delta CT}$ for each sample, and the results are expressed as the group mean fold change ± SD.

Table 1. Primer Sequences for the Mouse Genes Characterized in this Experiment

Gene	Primer
GAPDH	Forward primer: 5′ TGTCCGTCGTGG ATCTGAC 3′ Reverse primer: 5′ CCTGCTTCACCA CCTTCTTG 3′
Substance P	Forward primer: 5′ AAGCCTCAGCAG TTCTTTGG 3′ Reverse primer: 5′ TCTGGCCATGTC CATAAAGA 3′
CGRP	Forward primer: 5′ TGCAGGACTATA TGCAGATGAAA 3′ Reverse primer: 5′ GGATCTCTTCTG AGCAGTGACA 3′
MOR	Forward primer: 5′ AGCCCTTCCATG GTCACA 3′ Reverse primer: 5′ GGTGGCAGTCTT CATTTTGG 3′

CGRP = calcitonin gene-related peptide; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MOR = μ-opioid receptor.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD). All the statistical parameters used in the experiments were calculated using GraphPad PRISM (version 5.0a; GraphPad Software, La Jolla, CA). The statistical significance of differences between groups was assessed with one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired Student *t* test (two-tailed). A *P* value of < 0.05 was considered statistically significant.

Results

Characterization of the Peritoneal Metastasis Model

To analyze the progression of peritoneal dissemination of tumor cells, luciferase gene-transfected 60As6 cells were implanted into the abdominal cavities of mice (10^6 cells/cavity). Photon-counting analyses were performed at the site of dis-

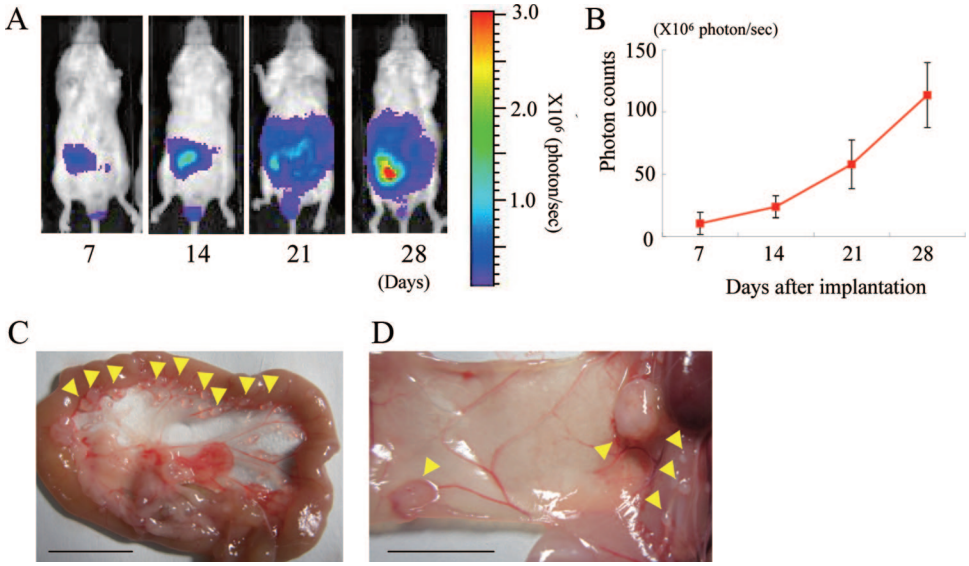

Fig. 1. Characterization of the peritoneal metastasis model. Photon-counting analysis of peritoneal dissemination after the intraperitoneal inoculation of 60As6 Luc cells. Scid mice bearing 60As6Luc tumors in the abdominal cavity were anesthetized and subjected to observations after intraperitoneal administration of luciferin (*A*). Quantitative analysis of the progression of peritoneal disseminated metastasis of tumor cells (n = 5). This experiment was repeated three times, and similar results were observed each time (*B*). Macroscopic evidence of peritoneal dissemination in scid mice bearing tumor cells. There were metastatic lesions in the intestine (*C*, *arrowheads*) and the parietal peritoneum (*D*, *arrowheads*). Scale bar: 10 mm.

semination at intervals of 7 days. Figure 1A shows a typical example. This method made it possible to observe the same animals over time. A tumor growth curve that reflected the progression of peritoneal dissemination was obtained by plotting the number of photons *versus* time (fig. 1B, n = 5). Dissemination to the mesenterium and parietal peritoneum was noted based on the macroscopic appearance of peritoneal dissemination in mice 28 days after inoculation (fig. 1, C and D, *arrowheads*).

Hypersensitivity to Mechanical Stimulation and Visceral Pain-related Behavior Induced by Peritoneal Carcinomatosis in Mice

The hypersensitivity to mechanical stimulation in the abdomen was quantified by counting the number of withdrawal behaviors in response to stimulation with von Frey filaments 14 and 28 days after the inoculation of tumor cells (fig. 2A). Twenty-eight days after inoculation, but not 14 days after inoculation, mice with peritoneal dissemination showed a significant increase in the nociceptive score in response to mechanical stimulation ($P < 0.05$ *vs.* control group, one-way ANOVA followed by the Bonferroni multiple comparisons test, control group: n = 5, tumor group: n = 7). We next examined visceral pain-related behavior, which was assessed in terms of the degree of hunching and the time spent hunching (over 180 s). Hunching behavior has been described previously as a measure of abdominal pain caused by pancreatic cancer in mice.[14] Visceral pain-related behavior was examined at 14 and 28 days after the inoculation of tumor cells (fig. 2B). Spontaneous visceral pain-related behavior became evident at 28 days after inoculation ($P < 0.05$ *vs.* control group, one-way ANOVA followed by the Bonferroni multiple comparisons test, each group: n = 5).

Changes in c-Fos-positive Cells in the Dorsal Horn of the Spinal Cord Induced by Peritoneal Carcinomatosis

Spinal cord tissue samples were obtained from animals 28 days after tumor inoculation and from control mice. In control mice, few cFos-positive cells were found in the superficial layer (L1–2 laminae) or deep layer (L3–5 laminae) of the dorsal horn (fig. 3A). In tumor-bearing mice, the number of c-Fos-positive cells was significantly increased in both the superficial and deep layers of the dorsal horn (fig. 3, B and C, $P < 0.05$ *vs.* control group, one-way ANOVA followed by the Bonferroni multiple comparisons test, each group: n = 5).

Changes in the Expression of Substance P, CGRP, and MOR Induced by Peritoneal Carcinomatosis in Mice

Twenty-eight days after inoculation, the mRNA concentration level of substance P was significantly increased in the DRG of tumor-bearing mice compared with that in control mice, whereas there was no significant difference at 14 days after inoculation (fig. 4A, $P < 0.05$ *vs.* control group, one-way ANOVA followed by the Bonferroni multiple comparisons test, each group: n = 7). In addition, the mRNA concentrations of CGRP in the DRG of tumor-bearing mice were not different from those in the control mice at 14 and 28 days after inoculation (fig. 4B). Under these conditions, 28 days after inoculation, but not 14 days after inoculation, the mRNA concentration of MOR was significantly decreased in the DRG of tumor-bearing mice compared with

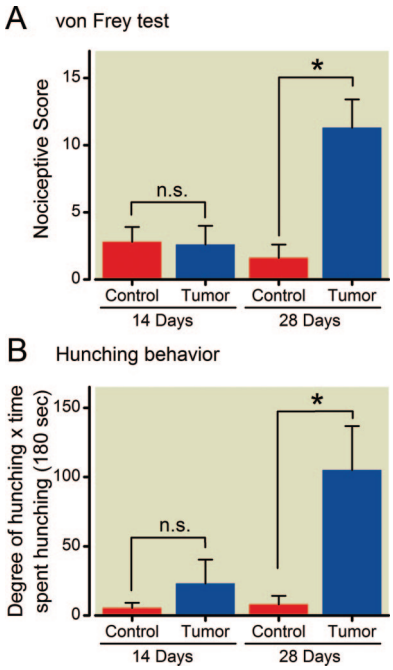

Fig. 2. Abdominal hypersensitivity to mechanical stimulation and visceral pain-related behavior induced by peritoneal carcinomatosis in mice. Hypersensitivity was quantified by counting the number of withdrawal behaviors in response to mechanical stimulation at 14 and 28 days after the inoculation of tumor cells (*A*). Visceral pain-related behavior was assessed in terms of the degree of hunching and time spent hunching (over 180 s) at 14 and 28 days after the inoculation of tumor cells (*B*). Each column represents the mean ± SD. * $P <$ than 0.05 *versus* control group, one-way ANOVA followed by the Bonferroni multiple comparisons test.

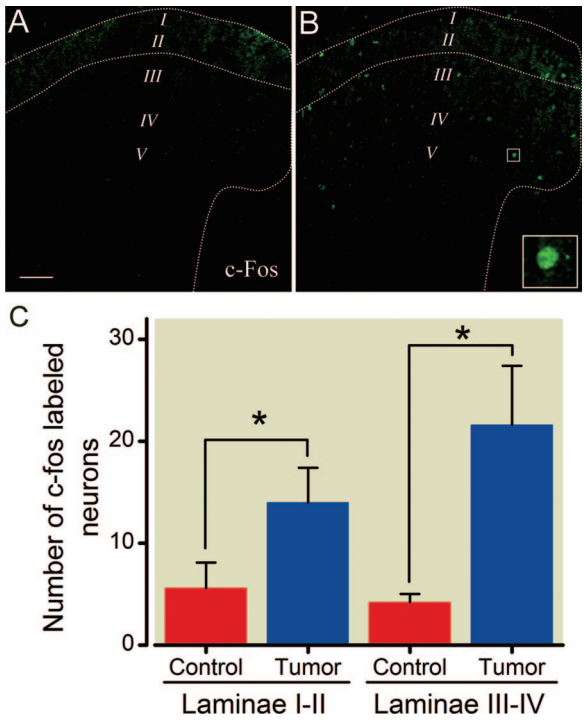

Fig. 3. Changes in c-Fos-positive cells in the dorsal horn of the spinal cord induced by peritoneal carcinomatosis. c-Fos expression in a control mouse (*A*) and in the dorsal horn of a tumor-bearing mouse on day 28. *Inset* is a high magnification image of the squared area (*B*). Increases in c-Fos-positive cells were observed in the superficial layer (L1–2 laminae) and the deep layer (L3–5 laminae) of the dorsal horn in tumor-bearing mice (*C*). The results represent the means ± SD. * $P < 0.05$ *versus* control group, one-way ANOVA followed by the Bonferroni multiple comparisons test. Scale bar: 50 μm.

that in control mice (fig. 4C, $P < 0.05$ *vs.* control group, one-way ANOVA followed by the Bonferroni multiple comparisons test, each group: n = 7).

Changes in MOR in Substance P-positive DRG Neurons Induced by Peritoneal Carcinomatosis in Mice

The expression patterns of MOR in DRG neurons were determined at 28 days after inoculation in control and tumor-bearing mice. In the T10–12 DRGs of control mice, MOR-positive profiles were seen in 39.0 ± 1.6% of all neurons (fig. 5A), which was significantly greater than the value in tumor-bearing mice (26.7 ± 2.9%, fig. 5, B and C, $P <$ 0.05 *vs.* control group, unpaired Student *t* test (two-tailed), each group: n = 3). In addition, the percentage of substance P-positive profiles in tumor-bearing mice (24.2 ± 3.6%) was significantly greater than that in control mice (15.4 ± 1.1%, fig. 5, D, E ,and F, $P < 0.05$ *vs.* control group, unpaired Student *t* test (two-tailed), each group: n = 3). We next investigated the change in the colocalization of MOR with substance P (*arrowhead*). Although 69.6 ± 4.9% of substance P-positive profiles in control mice were also MOR-positive, tumor inoculation significantly decreased this percentage (38.7 ± 0.9%, fig. 5G, $P < 0.05$ *vs.* control group, unpaired Student *t* test [two-tailed], each group: n = 3).

Effect of Systemic Administration of Morphine on the Nociceptive Behavior in Response to Mechanical Stimulation in Tumor-bearing Mice, Caerulein-injected Mice, and CFA-injected Mice

Repeated treatment with caerulein (six injections, 50 μg/kg) resulted in abdominal hypersensitivity to mechanical stimulation, indicating that caerulein-injected mice exhibited an acute pancreatitis pain-like state.[19,20] On the other hand, the latency of paw withdrawal induced by mechanical stimulation was reduced dramatically by the intraplantar injection of a CFA solution into the mouse hind paw.[21] In tumor-bearing, caerulein-injected, and CFA-injected mice, subcutaneous injection of saline did not have any effect on the nociceptive score. In caerulein-injected and CFA-injected mice, subcutaneous injection of morphine reduced the nociceptive score in a dose-dependent manner in response to mechanical stimulation compared with that in mice injected with saline ($P < 0.05$, 1 mg/kg: 68.4 ± 17.5% of the basal value, 3 mg/kg: 43.8 ± 21.4% of the basal value, 5 mg/kg: 22.2 ± 10.1% of the basal value, caerulein-saline group [n = 5] *vs.* caerulein-morphine group [n = 6]; $P < 0.05$, 3 mg/kg: 42.9 ± 13.1% of the basal value, 5 mg/kg: 9.5 ± 14.9% of the basal value, CFA-saline group [n = 6] *versus* CFA-mor-

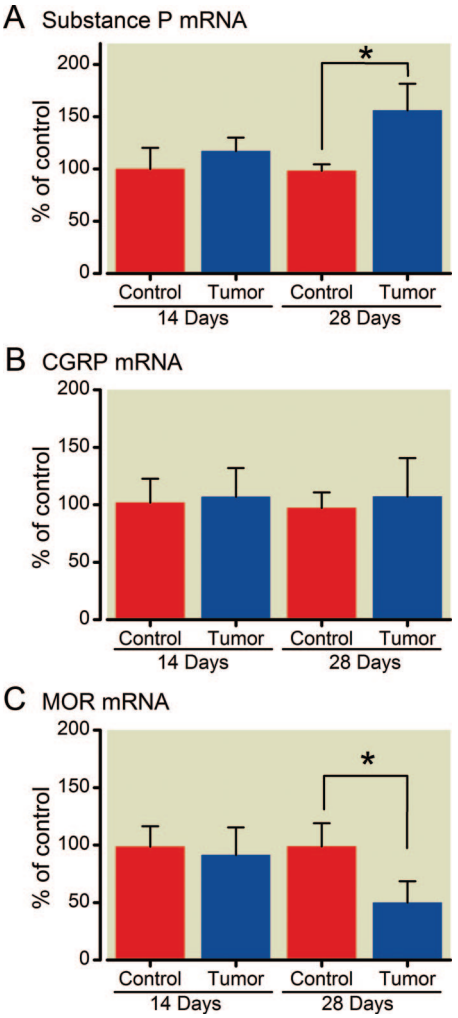

Fig. 4. Transcriptional regulation of substance P, calcitonin gene-related peptide (CGRP), and μ-opioid receptor (MOR) in the dorsal root ganglia (DRG) induced by peritoneal carcinomatosis. Expression of substance P (*A*), CGRP (*B*), and MOR (*C*) in the DRG of tumor-bearing mouse on days 14 and 28. Real-time reverse transcription polymerase chain reaction was carried out on messenger RNA (mRNA) obtained from thoracic 6–13 th and L1–3 DRG. The mRNA concentrations were normalized to those for glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene), and the results are presented as the means ± SD. * $P < 0.05$ *versus* control group, one-way ANOVA followed by the Bonferroni multiple comparisons test.

phine group [n = 7], one-way ANOVA followed by the Bonferroni multiple comparisons test). In tumor-bearing mice, subcutaneous injection of morphine at a dose of 3 mg/kg, which significantly reduced the nociceptive score in both caerulein-injected and CFA-injected mice, did not have any effect on the nociceptive score. In contrast, higher doses of morphine (5 and 10 mg/kg) produced a significant reduction in the nociceptive score ($P < 0.05$, 52.8 ± 15.6% and 39.2 ± 9.3% of the basal value, respectively, tumor-saline group [n = 5] *vs.* tumor-morphine group [n = 5], one-way ANOVA followed by the Bonferroni multiple comparisons test). However, the effect of morphine was less in tumor-bearing mice than in caerulein-injected and CFA-injected mice (fig. 6).

Discussion

Patients with cancer in the advanced stages, especially those with bone metastasis and cancerous peritonitis, endure significant pain. Patients with tumors involving bone destruction and nerve damage are particularly likely to experience severe pain.[27–29] Although published guidelines for pain management are available, the routine use of this treatment does not always alleviate this kind of pain.[3,29,30] Cancer pain often is treated by higher doses of morphine, which can be accompanied by side effects, including sedation, respiratory depression, and interference with gastrointestinal motility, and often provides only incomplete relief. Not surprisingly, novel, more effective analgesics are needed for the treatment of severe opioid-resistant cancer pain, such as abdominal pain caused by cancerous peritonitis.

In this study, we developed a novel mouse model for abdominal pain caused by cancerous peritonitis. Generally, it is difficult to monitor growth and subsequent progression of tumors to cancerous peritonitis, unlike with subcutaneous tumors. In the current study, we observed the progression of dissemination in real-time using luciferase gene-transfected cells and an *in vivo* photon-counting analysis. The level of emitted photon intensity gradually increased over time after tumor cell inoculation, and the intensity level at 28 days after tumor inoculation was approximately 10-fold that at 7 days. Mice with peritoneal dissemination showed dramatic increases in both their nociceptive scores in response to mechanical stimulation and visceral pain-related behavior at 28 days after the inoculation of tumor cells. However, these pain-like behaviors were not observed 14 days after inoculation, suggesting that this abdominal hypersensitivity is observed predominantly in mice at the relatively late stage of cancerous peritonitis.

Nociceptors relay information from the periphery to the spinal cord, where they target secondary neurons in the superficial (L1–2 laminae) and deep layers (L3–5 laminae) of the dorsal horns.[31] Some of these secondary neurons are projection neurons that then pass this nociceptive information to the central nervous system.[31] To visually identify the changes in the activity of spinal neurons related to pain in this model, we observed the changes in the immunoreactivity of c-Fos, an immediate-early gene that is widely used in pain research as a marker for neuronal activation.[32–34] At 28 days after inoculation, there was an increase in c-Fos-positive cells in both the superficial and deep layers of the spinal cord. Second-order neurons in the spinal cord that receive visceral afferent input are located principally in the superficial spinal laminae, deeper in L5 and L10 laminae. Some studies have shown that somatic structures send afferents to L1–3 laminae and to deep L4 and L5 laminae.[35,36] Taken together, the current findings suggest that the progressive insult of the

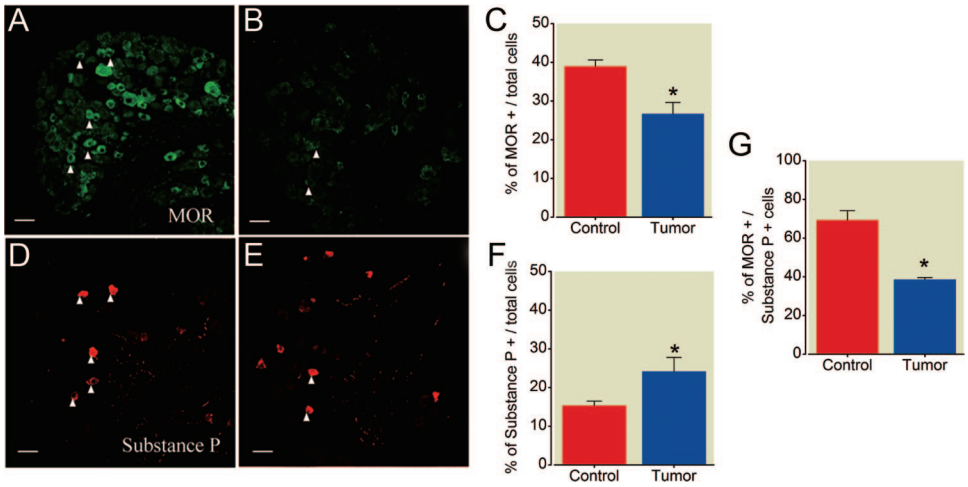

Fig. 5. Expression of μ-opioid receptor (MOR) in the dorsal root ganglia (DRG) of a control mouse (*A*) and a tumor-bearing mouse (*B*). Expression of substance P in the DRG of a control mouse (*D*) and a tumor-bearing mouse (*E*). Percentage of total cells expressing MOR (*C*), substance P (*F*), or MOR/substance P (*G*) in the DRG of control mice and tumor-bearing mice. The results are presented as the means ± SD. * $P < 0.05$ *versus* control group, unpaired Student *t* test (two-tailed). Scale bar: 20 μm.

peripheral nerve on the mesenterium and parietal peritoneum caused by tumor growth may result in the progression of wide-ranging pain accompanied by an increase in c-Fos-positive cells in the spinal cord.

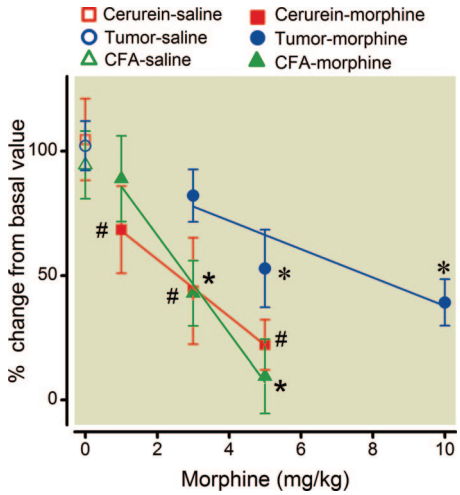

Fig. 6. Effects of systemic morphine treatment on the nociceptive scores in tumor-bearing (*circles*), caerulein-injected (*squares*), or complete Freund's adjuvant (CFA)-injected mice (*triangles*). Six hours after the last injection of caerulein (six injections, 50 μg/kg) or 3 days after CFA-injection, groups of mice were treated with subcutaneous morphine (1, 3, and 5 mg/kg). Twenty-eight days after the inoculation of tumor cells, groups of mice were treated with subcutaneous morphine (3, 5, and 10 mg/kg). The behavioral test was performed 30 min after the injection of morphine. Hypersensitivity to mechanical stimulation was quantified by counting the number of withdrawal behaviors in response to mechanical stimuli. The results are presented as the means ± SD. # $P < 0.05$, caerulein-saline group *versus* caerulein-morphine group. * $P < 0.05$, CFA-saline group *versus* CFA-morphine group. * $P < 0.05$, tumor-saline group *versus* tumor-morphine group, one-way ANOVA followed by the Bonferroni multiple comparisons test.

A variety of neurochemical and other markers have been used to characterize sensory neurons as nociceptive. The neuropeptides substance P and CGRP are considered to be markers of putative nociceptive DRG neurons.[31] In the current model, we observed a dramatic increase in the expression of substance P but not CGRP in the DRG of mice with peritoneal dissemination. Consistent with the data, the percentage of substance P-positive neurons apparently was increased in tumor-bearing mice. Substance P and CGRP have been reported to be up-regulated in both sensory neurons and primary afferent terminals in models of inflammatory pain.[37,38] In contrast, these neurotransmitters have been shown to be down-regulated in the spinal cord of models of nerve injury.[39,40] There was no significant change in the expression of these peptides in a murine model of bone cancer pain.[41] Substance P is abundant in visceral primary afferents (more than 80%),[42] and neurokinin1 receptor knockout mice show a significant deficit in visceral nociceptive perception.[43,44] Furthermore, inflammatory cytokine and neurotropic factors can induce the increased production of substance P and neurokinin1 receptors.[45] These data support the idea that inflammatory cytokine and neurotropic factors released by damaged peripheral nerve and/or tumor cells may promote the novel synthesis of substance P in the DRG of mice with peritoneal carcinomatosis. This phenomenon would correspond to the abdominal pain caused by the peritoneal dissemination of cancer cells in mice.

Another key finding in the current study was that peritoneal carcinomatosis decreased the mRNA expression of MOR in the DRG. In addition to MOR mRNA, the results of an immunohistochemical analysis revealed that the percentage of substance P-positive neurons that also were MOR-positive was decreased remarkably in the DRG of tumor-bearing mice. In relation to the down-regulation of MOR in the DRG, the dose–response curve for the antihy-

peralgesic effect of morphine against tumor-dependent pain was shifted to the right compared with that found in mice with either caerulein-induced visceral pain or CFA-induced inflammatory pain. Changes in MOR expression in the DRG have been shown in several pain models. Models of peripheral nerve injury have shown a dramatic decrease in MOR expression in the DRG, and such injuries reduce the effects of MOR agonists.[46–49] On the other hand, peripheral inflammation increases MOR expression in the dorsal horn of the spinal cord and the DRG, which are responsible for enhancing the effects of MOR agonists.[50,51] Luger *et al.*[52] and Yamamoto *et al.*[25] showed that higher doses of morphine were required to treat bone cancer pain than to treat inflammatory pain. It has been proposed that an increase in MOR mRNA production increases an opioid's efficacy at individual nociceptors.[53] Taken together, the current findings strongly support the idea that the deceased MOR expression in substance P-positive DRG may correspond to the reduction in the morphine-induced antinociception or analgesia in mice with peritoneal carcinomatosis.

In conclusion, mice with peritoneal carcinomatosis exhibit hypersensitivity to mechanical stimulation and visceral pain-like behavior, which is accompanied by the up-regulation of substance P and the down-regulation of MOR. This newly developed model may be important for studying the pathogenesis of abdominal pain caused by cancerous peritonitis.

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