[**Original Research**]

The κ-Opioid Receptor Agonist Nalfurafine Enhances the Chemotherapy-induced Survival Advantage in Pancreatic Cancer-bearing Mice

Yusuke HAMADA[*1], Yoshihiko TASAKI[*1], Kana MORITA[*1], Kohei YAMAMIZU[*2, *3], Michiko NARITA[*1], Fukiko MATSUYAMA[*1, *4], Masami SUZUKI[*5], Daigo IKEGAMI[*1], Kazuhiko ARAKAWA[*1], Yasuyuki NAGUMO[*6], Miho KAWATA[*1], Yasuhito UEZONO[*7], Hiroshi NAGASE[*6], Kazunori AOKI[*8], Jun K YAMASHITA[*2, *3], Naoko KUZUMAKI[*1], and Minoru NARITA[*1, *9]

[*1] Department of Pharmacology, Hoshi University School of Pharmacy and Pharmaceutical Sciences
[*2] Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University
[*3] Laboratory of Stem Cell Differentiation, Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University
[*4] Japan Small Animal Medical Center
[*5] Division of Cancer Genomics, National Cancer Center Research Institute
[*6] Department of Medicinal Chemistry, International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba
[*7] Division of Cancer Pathophysiology, National Cancer Center Research Institute
[*8] Division of Molecular and Cellular Medicine, National Cancer Center Research Institute
[*9] Life Science Tokyo Advanced Research Center (L-StaR), Hoshi University School of Pharmacy and Pharmaceutical Sciences

(Accepted November 21, 2016)

Abstract: Pancreatic cancer is a leading cause of cancer-related deaths; it shows poor survival rates and a poor prognosis. Therefore, more effective therapies for pancreatic cancer are needed in clinical practice. In this study, we demonstrated that the κ-opioid receptor agonist nalfurafine dramatically facilitated the gemcitabine-induced survival advantage in pancreatic cancer-bearing mice. Additionally, *in vitro* treatment with nalfurafine significantly inhibited vascular tube formation, but not cell proliferation in Pan02 cells. These findings suggest that nalfurafine may be useful for enhancing the chemotherapy-induced survival advantage for pancreatic cancer by directly suppressing tumor angiogenesis.

Key words: κ-Opioid receptor, Nalfurafine, Tumor angiogenesis, Pancreatic cancer, Chemotherapy

INTRODUCTION

Numerous clinical observations have indicated that advanced pancreatic cancer with peritoneal dissemination is only slightly influenced by systemic chemotherapy and its prognosis remains poor. Thus, the occurrence of peritoneal dissemination in pancreatic cancer patients is regarded as a terminal condition[1, 2]. Accordingly, more effective therapies for pancreatic cancer are needed to improve therapeutic responses and increase survival in pancreatic cancer patients.

Tumor angiogenesis is a key event in tumor progression and metastasis that enables cancer cells to intake nutrients and oxygen and leads to the acquisition of mobility and invasiveness[3, 4]. There is a growing body of evidence that the activation of several angiogenesis-related factors, such as vascular endothelial growth factor (VEGF), in the tumor microenvironment can cause tumor angiogenesis and aggravation, suggesting that anti-angiogenic therapies could be an important approach to cancer treatment[5, 6]. In clinical medicine, it has been generally accepted that an anti-angiogenic agent (e.g., bevacizumab, a humanized monoclonal immunological sequestering VEGF-A) should be administered in combination with various chemotherapies to enhance their effects[7].

Opioid systems mainly consist of three different types

Corresponding author: Minoru Narita, Ph.D., Department of Pharmacology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2–4–41, Ebara, Shinagawa-ku, Tokyo 142–8501, Japan
E-mail: narita@hoshi.ac.jp

of opioid receptors, μ, δ, and κ (MOR, DOR, and KOR), and the respective endogenous peptide. These opioid systems regulate a wide range of physiological functions such as pain, the emotional response, and the reward circuitry in neural tissues[8, 9]. Clinically, opioid analgesics such as morphine, a MOR agonist, have been broadly applied to relieve pain associated with all types of cancer. However, independent studies have shown that morphine can either decrease or increase tumor growth in mice[10, 11], and the effects of opioids on tumor growth are still unclear. In our recent studies, we first found that κ-opioid peptides acted as novel anti-angiogenic modulators by suppressing the expression of VEGF receptors during vascular differentiation in development and tumor growth[12–14]. In addition, nalfurafine, a novel KOR agonist, was recently synthesized in Japan[15] and has been clinically approved for use in hemodialysis-related uremic pruritus.

In this study, we demonstrated whether nalfurafine could enhance the chemotherapy-induced survival advantage in pancreatic cancer-bearing mice.

MATERIALS AND METHODS

1. Animals

This study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The experiments were performed on male C57BL/6J mice (Tokyo Laboratory Animals Science, Tokyo, Japan), aged 6 weeks at the start of the experimental procedures. All animals were housed 5 per cage and kept in a room with an ambient temperature of 23±1℃ and a 12 h light-dark cycle (lights on 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum during the experimental period and behavioral testing occurred in the morning.

2. Pancreatic cancer cell inoculation

Pan02 cells, a murine pancreatic ductal adenocarcinoma (PDAC) cell line, were cultured and prepared as described previously[16]. The density of Pan02 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. In the control group, PBS was injected into the abdominal cavity instead of Pan02 cells. Body weight and food consumption were measured 12 and 24 days after tumor implantation.

Hunching behavior was examined as described previously[17, 18]. Mice were placed individually in the center of an open field arena and observed for 300 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.[17]: 0: normal coat luster, displays exploratory behavior; 1: mild rounded-back posture, normal coat luster, displays slightly reduced exploratory behavior; 2: severe rounded-back posture, displays considerably reduced exploratory behavior, piloerection, intermittent abdominal contractions. Behavioral testing was performed at 12 and 24 days after tumor inoculation.

3. Survival studies

Beginning 7 days after tumor inoculation, mice were injected intraperitoneally with saline, gemcitabine (GEM; 100 mg/kg, *bis in 7d*; Wako Pure Chemical Industries, Ltd., Osaka, Japan), nalfurafine (NAL; 10 μg/kg, *b.i.d.*; TORAY, Tokyo, Japan) and GEM/NAL for 6 weeks. The survival time of each group was calculated from the date of enrollment to the date of death from tumor inoculation by the Kaplan-Meier method.

4. Cell viability assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. In the MTT assay, Pan02 cells (5×10^3 cells/well) were cultured in a 96-well plate and then treated with gemcitabine (0.01−1 μM) or nalfurafine (0.01−1 μM) 24 h after the cell culture. Forty-eight hours after drug treatments, 20 μl of MTT solution (5 mg/ml, Sigma-Aldrich Co., St. Louis, MO, USA) was added to each well of the culture medium. After incubation for another 2 h, the medium was removed, and 100 μl of dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd.) was added to resolve formazan crystals. Optical density was measured using a luminometer (Glomax, Promega Co., Madison, WI, USA) at an absorption wavelength of 600 nm (test wavelength) and 750 nm (reference wavelength). In each experiment, three replicates were prepared for each sample. The proportion of living cells was determined based on the difference in absorbance between samples and controls.

5. Tube formation assay

Human umbilical vein endothelial cells (HUVECs; Lonza Group, Ltd., Basel, Switzerland) were prepared as described previously[13]. In the tube formation assay, HUVECs (1.5×10^4 cells/well) were cultured in a 24-well plate coated with 150 μl Matrigel Basement Membrane Matrix GFR (BD Biosciences, San Jose, CA, USA). The tube formation assay was performed under treatment with 10 μM nalfurafine.

6. Statistical analysis

Data are expressed as the mean with *SEM*. No data points were removed from the statistical analysis except as specified. The data were subjected to an unpaired *t*-test or one-way analysis of variance (ANOVA) test followed by the Bonferroni multiple comparisons test as appropriate for the experimental design. The data with time-dependent changes were analyzed using two-way repeated measures (RM) ANOVA followed by the Bonferroni post-hoc test, where appropriate. Mortality data were compared using Kaplan-Meier plots and the log-rank test. All statistical analyses were performed with Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

1. Characterization of pancreatic cancer-bearing mice

First, we generated a mouse model of pancreatic cancer with peritoneal metastasis by the intraperitoneal inoculation of murine pancreatic cancer Pan02 cells (Fig. 1A). To assess the effect of tumor inoculation, we observed the changes in body weight and food consumption in mice for 24 days after tumor inoculation. Both the body weight and food consumption in mice with tumor inoculation were significantly decreased compared to those in control mice (Fig. 1B, C; $^{**}p<0.01$, $^{***}p<0.001$ vs. Control). Next, we examined visceral pain-related behavior caused by intraperitoneal tumor inoculation in mice. Hunching behavior has been previously described as a measure of abdominal pain caused by pancreatic cancer in mice[17]. Here we demonstrated that marked spontaneous visceral pain-related behavior was observed in mice at 12 and 24 days after tumor inoculation com-

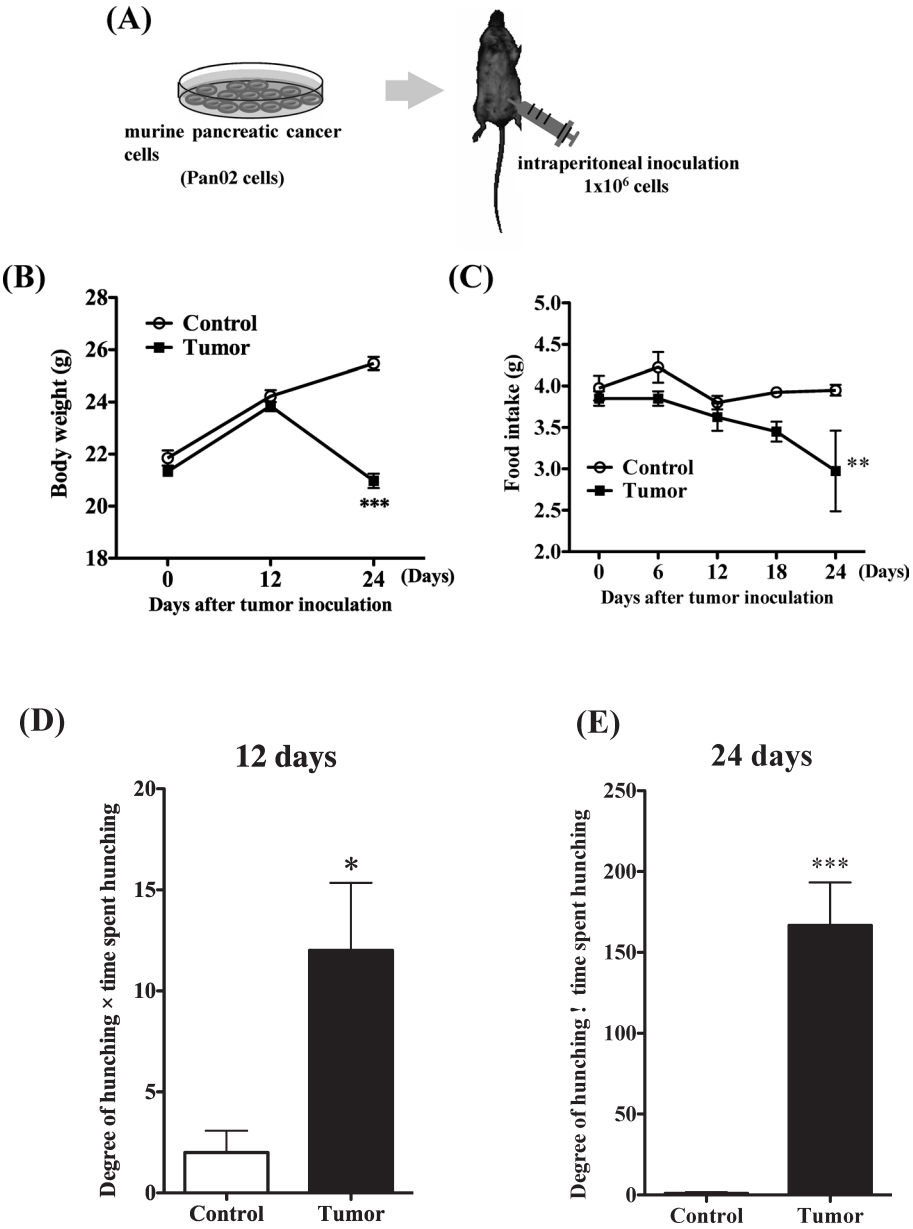


Fig. 1 Characterization of pancreatic cancer-bearing mice. (A) Schematic illustration of the intraperitoneal inoculation of Pan02 cells. (B, C) Time-course changes in body weight (B) and food consumption (C) of tumor-bearing mice after tumor inoculation. Each point represents the mean with *SEM* (n=4). Two-way RM ANOVA followed by the Bonferroni post-hoc test: $^{**}p<0.01$, $^{***}p<0.001$ vs. Control group. (D, E) Changes in visceral pain-related behavior of tumor-bearing mice in terms of the degree of hunching and time spent hunching (over 300 s) at 12 and 24 days after tumor inoculation. Each column represents the mean with *SEM* (n=4). Unpaired t-test: $^{*}p<0.05$, $^{***}p<0.001$ vs. Control group.

pared to that in control mice (Fig. 1D, E; $*p<0.05$, $***p<0.001$ vs. Control, unpaired t-test; Fig. 1D, E; $F_{(1,6)}=33.31$, $p=0.0012$ for interaction, Two-way RM ANOVA). Collectively, we hypothesized that these tumor-bearing mice showed peritoneal metastasis of pancreatic cancer.

2. Treatment with the KOR agonist nalfurafine enhanced the survival advantage of gemcitabine in pancreatic cancer-bearing mice

In general, gemcitabine is widely recommended as first-line chemotherapy against pancreatic cancer. On the other hand, we previously found that KOR agonists may be able to inhibit tumor growth[13, 14]. Therefore, to investigate the effect of the KOR agonist nalfurafine on the anti-tumor effect of gemcitabine, we examined whether combined treatment with gemcitabine and nalfurafine could prolong survival in a mouse model of pancreatic cancer with peritoneal metastasis according to the Kaplan-Meier method (Fig. 2A). As a result, we first confirmed that peritoneal metastasis-model mice that were injected with gemcitabine (100 mg/kg, *bis in 7d*) for 6 weeks from 7 days after tumor inoculation exhibited clearly prolonged survival compared with that in control mice (Fig. 2B, C; $***p<0.001$ vs. Control; proportion of median survival, 2.232; 95% CI, 1.826 to 2.638; an improvement of 42.5 days). In contrast, the repeated-intraperitoneal administration of only nalfurafine at the dose of 10 μg/kg (*b.i.d.*), which has shown its pharmacological effects[13], slightly prolonged survival in peritoneal metastasis-model mice (Fig. 2B, C; $*p<0.05$ vs. Control; proportion of median survival, 1.275; 95% CI, 0.859 to 1.692; an improvement of 9.5 days). Interestingly, we demonstrated that the survival time of mice that were treated with gemcitabine in combination with nalfurafine was significantly greater than that of mice treated with gemcitabine alone (Fig. 2B, C; $^{\#}p<0.05$ vs. GEM; proportion of median survival, 1.130; 95% CI, 0.774 to 1.486; an improvement of 10.0 days). Taken together, these results suggest that treatment with nalfurafine enhanced the survival advantage induced by treatment with gemcitabine in pancreatic cancer-bearing mice.

3. Treatment with nalfurafine suppresses tumor angiogenesis, but not cell growth, in tumor cells

To clarify the mechanisms that underlie the enhancement of the gemcitabine-induced survival advantage under combined treatment with nalfurafine in pancreatic cancer-bearing mice, we next investigated whether *in vitro* treatment with nalfurafine could directly suppress the growth of Pan02 cells using MTT assays. In this study, treatment with gemcitabine (0.01–1 μM) significantly suppressed the growth of Pan02 cells in a concentration-dependent manner (Fig. 2D; $***p<0.001$ vs. Control), whereas the growth of Pan02 cells was not affected by treatment with nalfurafine (0.01–1 μM) (Fig. 2E).

We next examined whether *in vitro* treatment with nalfurafine could directly inhibit angiogenesis using HUVEC tube formation assays. As a result, treatment with 10 μM nalfurafine dramatically suppressed HUVEC tube formation (Fig. 2F).

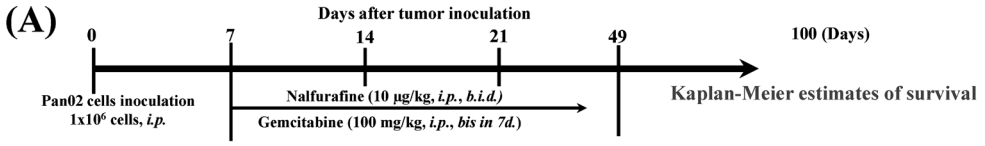
DISCUSSION

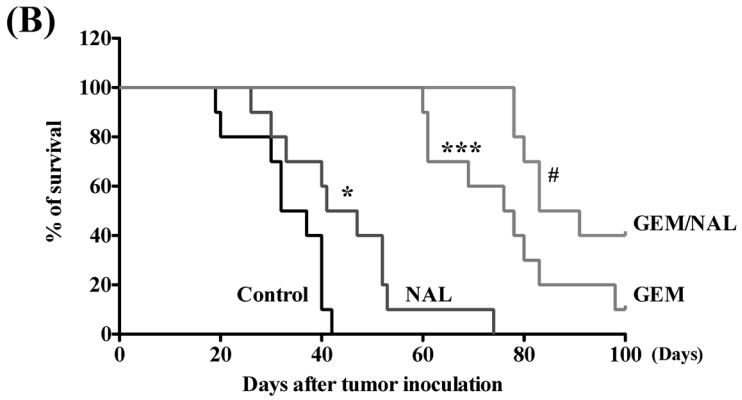
PDAC is known to be a leading cause of cancer-related deaths with poor survival rates and a poor prognosis[1, 2, 19]. Thus, more effective therapies are needed. In this study, we demonstrated that the KOR agonist nalfurafine could have potential for facilitating the chemotherapy-induced survival advantage in pancreatic cancer-bearing mice.

A large number of patients with PDAC are incredibly refractory and show cachexia and systemic metastasis[20, 21]. There has been little improvement in patient outcomes, even though considerable effort has been directed at optimizing the use of chemotherapy (e.g., gemcitabine) for pancreatic cancer[19, 22, 23]. First, we generated peritoneal metastasis-model mice by the intraperitoneal inoculation of Pan02 cells into their abdominal cavity. Next, we confirmed that these model mice exhibited weight loss, feeding suppression and spontaneous visceral pain-related behaviors induced by tumor inoculation, consistent with the description of clinical PDAC patients.

On the other hand, it has been widely accepted that tumor angiogenesis is crucial for tumor progression and metastasis, so that an anti-angiogenic agent is often included in the chemotherapy regimen in various cancer treatments[5, 6]. Especially, there is a growing body of evidence that VEGF, which is a critical factor in tumor angiogenesis, is highly expressed in the tumor microenvironment. As a result, it has been targeted for anticancer therapy using antibody drugs such as bevacizumab[7, 24]. In our recent studies, we clarified that endogenous κ-opioid peptides acted as novel anti-angiogenic modulators by inhibiting VEGF signaling during vascular differentiation in development and tumor progression, which occurs through the suppression of VEGF receptor expression, VEGFR2 and Neuropilin1, via the inhibition of cAMP/PKA signaling[12–14]. In addition, we previously demonstrated that tumor growth was dramatically suppressed by the repeated administration of KOR agonists in xenograft mice[13]. Therefore, in this study, we investigated whether the combined administration of gemcitabine and nalfurafine, which is a KOR agonist in widespread clinical use, could prolong survival in pancreatic cancer-bearing mice. We found that the administration of nalfurafine significantly enhanced the gemcitabine-induced survival advantage in pancreatic cancer-bearing mice.

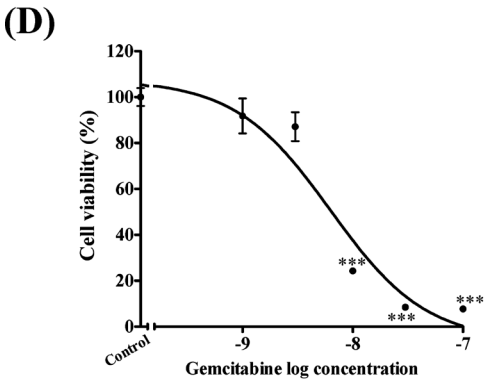
To clarify the underlying mechanism of the effect of nalfurafine on the gemcitabine-induced survival advantage, we next investigated whether treatment with nalfurafine could directly inhibit the growth of Pan02 cells. Unexpectedly, we found that *in vitro* treatment with gemcitabine significantly suppressed the growth of Pan02 cells, while there were no changes with nalfurafine. Finally, we confirmed that *in vitro* treatment with nal-

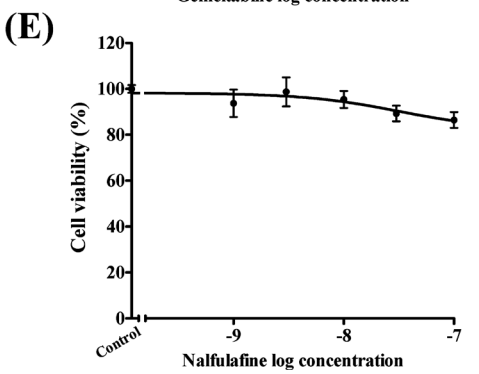




(C)

	Median survival	Hazard ratio	95% CI
Control	34.5 days	-	-
NAL	44.0 days	0.259 (vs Cont)	0.084 to 0.793
GEM	77.0 days	0.041 (vs Cont)	0.010 to 0.162
GEM/NAL	87.0 days	0.314 (vs GEM)	0.103 to 0.957





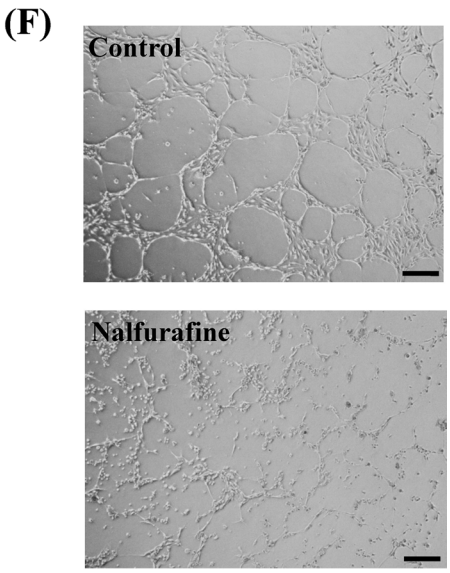


Fig. 2 Effects of the combined treatment with gemcitabine and nalfurafine on survival rates in pancreatic cancer-bearing mice. (A) Protocol for the combined treatment with gemcitabine (GEM, 100 mg/kg) and nalfurafine (NAL, 10 μg/kg) in pancreatic cancer-bearing mice. (B) Survival curves of pancreatic cancer-bearing mice treated with saline (Control), GEM, NAL and GEM/NAL. (n=10, Log-rank test: $^{*}p<0.05$, $^{***}p<0.001$ vs. Control group, #$p<0.05$ vs. GEM group). (C) The median survival and hazard ratio in Kaplan-Meier plots of pancreatic cancer-bearing mice treated with saline (Control), GEM, NAL or GEM/NAL. (D, E) Changes in cell viability of Pan02 cells under treatment with gemcitabine (D, 0.01–1 μM) and nalfurafine (E, 0.01–1 μM). Each data point represents the mean with *SEM* (n=5). One-way ANOVA was followed by the Bonferroni multiple comparisons test: $^{***}p<0.001$ vs. Control group. (F) Representative photographs of vasculature in the HUVEC tube formation assay under treatment with 10 μM nalfurafine. Scale bars: 200 μm.

furafine dramatically inhibited HUVEC tube formation. These results indicate that the suppression of tumor angiogenesis caused by the administration of nalfurafine occurred via the inhibition of VEGF signaling along with the activation of KORs in host endothelial cells in the tumor.

Taken together, these findings suggest that nalfurafine suppressed tumor angiogenesis, but not cell growth in tumor, during tumor progression, leading to enhancement of the gemcitabine-induced survival advantage in pancreatic cancer-bearing mice. These findings may suggest a novel strategy for chemotherapy.

Conflict of Interest

The authors declare no competing financial interests.

Acknowledgments

We would like to thank K. Torigoe, Y. Suda, C. Iwasawa, and D. Sato for their help with the experiments. This study was supported by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities (No. S1411019).

REFERENCES

1) Siegel R, Ma J, Zou Z, and Jemal A. Cancer statistics, 2016. CA. Cancer. J. Clin. 2016; 66: 7–30.
2) Michaud DS. Epidemiology of pancreatic cancer. Minerva chirurgica 2004; 59: 99–111.
3) Folkman J. Tumor angiogenesis: Therapeutic implications. N. Engl. J. Med. 1971; 285: 1182–1186.
4) Carmeliet P and Baes M. Metabolism and therapeutic angiogenesis. N. Engl. J. Med. 2008; 358: 2511–2512.
5) Bergers G and Benjamin LE. Tumorigenesis and the angiogenic switch. Nat. Rev. Cancer 3, 2003; 401–410.
6) Goel HL and Mercurio AM. VEGF targets the tumour cell. Nat. Rev. Cancer 2013; 13: 871–882.
7) Ferrara N, Hillan KJ, Gerber N, et al. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat. Rev. Drug Discov. 2004; 3: 391–400.
8) Kieffer BL and Gaveriaux-Ruff C. Exploring the opioid system by gene knockout. Prog. Neurobiol. 2002; 66: 285–306.
9) Narita M, Funada M, and Suzuki T. Regulations of opioid dependence by opioid receptor types. Pharmacol. Ther. 2001; 89: 1–15.
10) Tegeder I, Grosch S, Schmidtko A, et al. G protein-independent G1 cell cycle block and apoptosis with morphine in adenocarcinoma cells: Involvement of p53 phosphorylation. Cancer Res. 2003; 63: 1846–1852.
11) Gupta K, Kshirsagar S, Chang L, et al. Morphine stimulates angiogenesis by activating proangiogenic and survival-promoting signaling and promotes breast tumor growth. Cancer Res. 2002; 62: 4491–4498.
12) Yamamizu K, Furuta S, Katayama S, et al. The kappa opioid system regulates endothelial cell differentiation and pathfinding in vascular development. Blood 2011; 118: 775–785.
13) Yamamizu K, Furuta S, Hamada Y, et al. κ Opioids inhibit tumor angiogenesis by suppressing VEGF signaling. Sci. Rep. 2013; 3: 3213.
14) Yamamizu K, Hamada Y, and Narita M. κ Opioid receptor ligands regulate angiogenesis in development and in tumours. Br. J. Pharmacol. 2015; 172: 268–276.
15) Tsuji M, Takeda H, Matsumiya H, et al. The novel kappa-opioid receptor agonist TRK-820 suppresses the rewarding and locomotor-enhancing effects of morphine in mice. Life Sci. 2001; 68: 1717–1725.
16) Narumi K, Miyakawa R, Ueda R, et al. Proinflammatory proteins S100A8/S100A9 activate NK cells via interaction with RAGE. J. Immunol. 2015; 194: 5539–5548.
17) Sevcik MA, Jonas BM, Lindsay TH, et al. Endogenous opioids inhibit early-stage pancreatic pain in a mouse model of pancreatic cancer. Gastroenterology 2006; 131: 900–910
18) Suzuki M, Narita M, Hasegawa M, et al. 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. Anesthesiology 2012; 117: 847–856.
19) Neesse A, Algul H, Tuveson DA, et al. Stromal biology and therapy in pancreatic cancer: A changing paradigm. Gut 2015; 64: 1476–1484.
20) Ronga I, Gallucci F, Riccardi F, et al. Anorexia-cachexia syndrome in pancreatic cancer: Recent advances and new pharmacological approach. Adv. Med. Sci. 2014; 59: 1–6.
21) Greco SH, Tomkotter L, Vahle AK, et al. TGF-β blockade reduces mortality and metabolic changes in a validated murine model of pancreatic cancer cachexia. PLoS One 2015; 10: e0132786.
22) Ueno H, Ioka T, Ikeda M, et al. Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. J. Clin. Oncol. 2013; 31: 1640–1648.
23) Beger HG, Rau B, Gansauge F, et al. Treatment of pancreatic cancer: Challenge of the facts. World J. Surg. 2003; 27: 1075–1084.
24) Pan Q, Chanthery Y, Liang WC, et al. Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. Cancer Cell 2007; 11: 53–67.