Table 2. Patient background

Background factors	$n = 40$
Sex: M/F	28 (70.0%)/12 (30.0%)
Age (years old)	67.1 ± 12.5
Under 40	1 (2.5%)
40s	3 (7.5%)
50s	7 (17.5%)
60s	7 (17.5%)
70s	17 (42.5%)
80 or older	5 (12.5%)
Underlying disease (including overlaps)	
Cancer of upper digestive organs	9 (22.5%)
Cancer of lower digestive organs	4 (10.0%)
Perforation of the digestive tract	10 (25.0%)
Acute pancreatitis	1 (2.5%)
Liver disease (liver failure, hepatic cirrhosis, other)	5 (12.5%)
Cardiovascular disease (abdominal aortic aneurysm, other)	5 (12.5%)
Other	6 (15.0%)
Surgical division (emergency/scheduled)	20 (50.0%)/20 (50.0%)
Cause of SIRS (including overlaps)	
Infection	18 (45.0%)
Surgical stress	26 (65.0%)
Pancreatitis	2 (5.0%)
Other	7 (17.5%)
C-reactive protein (mg/dl)	13.8 ± 8.7
Number of organs with failure other than the lungs	
0	17 (42.5%)
1	10 (25.0%)
2	9 (22.5%)
3	3 (7.5%)
Not investigable	1 (2.5%)

SIRS, systemic inflammatory response syndrome

Table 3. Respiratory function and management at the initiation of sivelestat sodium administration

	$n = 40$
PaO_2/F_IO_2	
≤100 mmHg	8 (20.0%)
100–200 mmHg	14 (35.0%)
200–300 mmHg	18 (45.0%)
Chest X-ray score	
0 point	10 (25.0%)
1 point	6 (15.0%)
2 points	14 (35.0%)
3 points	6 (15.0%)
4 points	4 (10.0%)
Respiratory management method	
Invasive positive pressure ventilation	36 (90.0%)
Non-invasive positive pressure ventilation	4 (10.0%)

8.3 (range 1–35) days. The survival rate after 28 days was 90.0% (36/40).

Analysis of the Background of the Surviving and Fatal Cases

The backgrounds at the initiation of SIV administration of patients who had survived versus those who had died by

Table 4. Treatments

	$n = 40$
Combination therapy (including overlaps)	
Steroids	13 (32.5%)
Antimicrobial agents	39 (97.5%)
Protease inhibitors	18 (45.0%)
Diuretics	14 (35.0%)
Hemocatharsis (endotoxin adsorption)	3 (7.5%)
Sivelestat sodium administration	
After SIRS onset	0.8 ± 1.8 days
Within 24h	29 (72.5%)
1–3 days	7 (17.5%)
4 days or later	4 (10.0%)
After the initiation of respiratory management	0.8 ± 2.0 days
Within 24h	29 (72.5%)
1–3 days	7 (17.5%)
4 days or later	4 (10.0%)
After the onset of respiratory dysfunction	0.6 ± 1.8 days
Within 24h	35 (87.5%)
1–3 days	1 (2.5%)
4 days or later	4 (10.0%)

Table 5. Patient background by outcome

Background factors	Survival (n = 36)	Death (n = 4)	P value
Sex: Male/female	25/11	3/1	1.0000[a]
Age (years)	66.4 ± 12.6	73.5 ± 11.2	0.2851[b]
Number of positive items for SIRS	2.6 ± 1.2	3.0 ± 1.2	0.5262[b]
PaO_2/F_IO_2 (mmHg)	184.6 ± 70.0	193.5 ± 100.7	0.8171[b]
Chest X-ray score	1.6 ± 1.3	3.0 ± 0.8	0.0310[b]
Number of impaired organs other than the lungs	0.8 ± 0.9	2.3 ± 0.5	0.0044[b]
C-reactive protein level (mg/dl)	13.0 ± 8.3	20.2 ± 10.4	0.1220[b]
Surgery (emergency/scheduled)	17/19	3/1	0.6050[a]
Steroid (treated/untreated)	11/25	2/2	0.5839[a]

[a]Fisher exact test
[b]Unpaired t-test

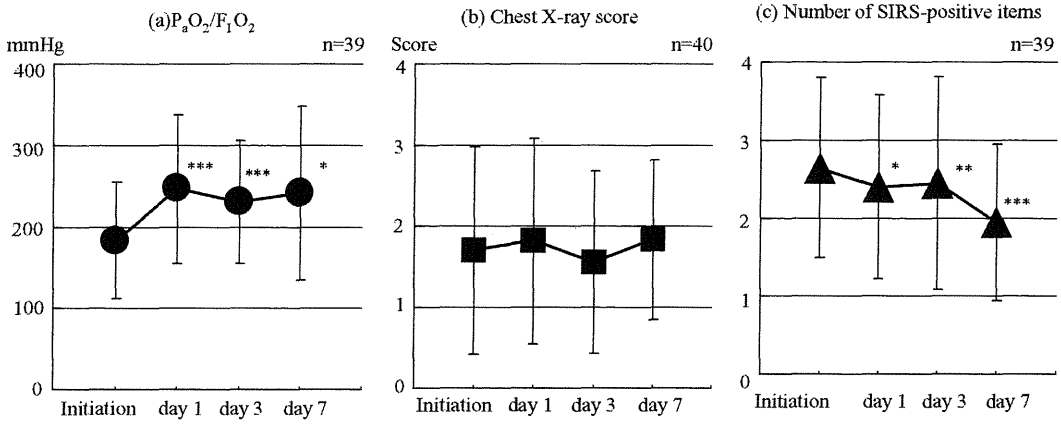

Fig. 1. Changes in the **a** P/F ratio, **b** chest X-ray score, and **c** number of positive items on the systemic inflammatory response syndrome (*SIRS*) criteria during the postoperative course. Wilcoxon signed-rank test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs initiation

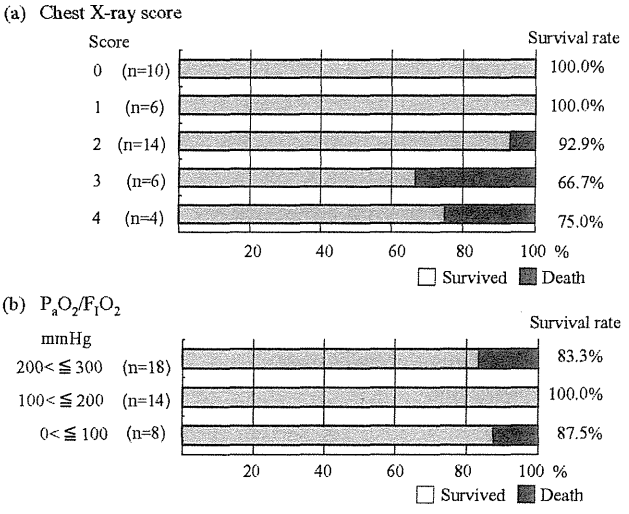

Fig. 2. Patient background by outcome in relation to **a** chest X-ray score and **b** P/F ratio. **a** Cochran–Armitage trend test, $P = 0.0308$; **b** Cochran–Armitage trend test, $P = 0.4917$

28 days after SIV administration are shown in Table 5 and Fig. 2. Of the patient background factors, the chest X-ray score was significantly higher in fatal cases in comparison to the patients who survived ($P = 0.0310$). The mean number of impaired organs other than the lungs was 0.8 in the surviving cases, but 2.3 in fatal cases, which was significantly higher ($P = 0.0044$). The increases in the chest X-ray score and mortality rate significantly correlated ($P = 0.0308$). The survival rate was 100% in cases in which the chest X-ray score was 0–1 (no infiltration or infiltration in one quadrant of one lung, if any), and 83.3% in cases in which the score was 2 or higher (apparent shadows were present). No significant correlation was noted between the increase in the P/F ratio and the mortality rate. The chest X-ray scores in surviving and fatal cases with a P/F ratio of 200–300 mmHg at the initiation of treatment were 1.1 ± 1.2 and 3.3 ± 0.6, respectively.

Discussion

Various factors, such as infection, may cause complications after surgery. The earliest acute complication that

can affect the prognosis is ALI/ARDS. Impairment of the lungs may lead to dysfunction of important organs, such as the liver and kidneys, due to a lack of oxygen supply, resulting in MOF. The mortality rate from ARDS has been reported to be 30%–65%,[10–13] indicating that treatment before the occurrence of ARDS or during the early stage of ARDS is important. Accordingly, various perioperative management methods, including respiratory management, have been investigated, and countermeasures have also been taken.

In Japan SIV, a selective neutrophil elastase inhibitor, became commercially available in 2002. Sivelestat sodium is administered to treat ALI/ARDS accompanying SIRS induced by various underlying diseases and factors, such as pneumonia[14] and sepsis,[14,15] and its usefulness has been confirmed in numerous clinical trials. Regarding postoperative SIV treatment, administration has also been reported after surgery for esophageal cancer[16–18] and cardiopulmonary bypass.[19] However, the clinical significance of SIV administration for patients with respiratory dysfunction following surgery is unclear.

We surveyed the effects of using SIV for postoperative respiratory dysfunction in patients who underwent surgery at the multiple facilities that make up our hospital to investigate the efficacy of SIV. Of the 40 cases included in our study, the P/F ratio was 200 mmHg or lower in 22 patients, and the chest X-ray score was 2 or higher in 24 patients, showing that this was a relatively ill patient population. This survey was performed in selected cases treated with SIV at multiple facilities during a 9-month period, and was not a comparative study with a control group. Therefore, it was not possible to completely clarify the usefulness of SIV in this study, as it is not known how untreated patients would have fared. However, the survival rate after 28 days (90.0%) was favorable, suggesting that postoperative multidisciplinary management, including SIV treatment, was superior to conventional postsurgical survival in patients with ARDS and SIRS. Since SIV was administered early after the occurrence of respiratory dysfunction, SIV may have contributed to the improvement of the prognosis.

When we stratified the patients according to outcome, the chest X-ray score and the number of impaired organs at the initiation of SIV administration correlated with the outcome, showing that these factors are related to the patient prognosis. In contrast, no correlation was noted between the P/F ratio and outcome.

In the USA–Europe joint AECC conference in 1994, the definition and diagnostic criteria for ALI were proposed.[20] Acute lung injury was defined as conditions with: (1) acute development, (2) a P/F ratio ≤300 mmHg, (3) bilateral infiltrations in the frontal view on chest radiography, and (4) pulmonary arterial wedge pressure ≤18 mmHg or the absence of clinical left arterial pressure elevation. However, microinfiltration that is only detectable by computed tomography scan may not be found by X-ray examination. Sixteen cases with a P/F ratio ≤300 mmHg and chest X-ray score of 1 or lower were included in this study. The early administration of SIV may have improved the prognosis in these cases, thus suggesting that intensive care, in accordance with that for ALI/ARDS patients, is already necessary for cases of SIRS with a P/F ratio of ≤300 mmHg.

We have herein demonstrated that the initiation of SIV administration in the early phase after the onset of respiratory dysfunction following surgery improves the prognosis for patients. Further investigations, including controlled clinical studies, will be necessary to fully determine the efficacy of using SIV for ALI/ARDS with SIRS following surgery.

Acknowledgments. We thank the following institutions for their participation in the investigation: Iizuka Hospital, Arita Kyoritsu Hospital, Oita Medical Center, Oita Red Cross Hospital, Kitakyushu Municipal Medical Center, Kyushu Medical Hospital, National Kyushu Cancer Center, Saiseikai Karatsu Hospital, Saiseikai Fukuoka General Hospital, Saiseikai Yahata General Hospital, Nakatsu Municipal Hospital, Nankai Hospital, Hita Central Hospital, Hiroshima Red Cross Hospital & Atomic Bomb Survivors Hospital, Fukuoka City Hospital, National Fukuoka-Higashi Medical Center, Beppu Medical Center, Matsuyama Red Cross Hospital, and Munakata Medical Association Hospital.

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Surg Today (2010) 40:1046–1049
DOI 10.1007/s00595-009-4155-x

SURGERY TODAY

Original Article

Curative Surgery Improves the Survival of Patients with Perforating Colorectal Cancer

KOTARO SHIBAHARA[1], HIROYUKI ORITA[2], TADASHI KOGA[2], HITOSHI KOHNO[2], HISANOBU SAKATA[3], YOSHIHIRO KAKEJI[1], and YOSHIHIKO MAEHARA[1]

[1]Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
[2]Department of Surgery, Saga Prefectural Hospital Koseikan, Saga, Japan
[3]Department of Surgery, Oita Prefectural Hospital, Oita, Japan

Abstract

Purpose. Colorectal cancers that manifest as a perforation are generally regarded as carrying a poor prognosis. We conducted this study to assess the outcome of colorectal cancer complicated by perforation.

Methods. Between 1996 and 2004, 848 patients underwent surgery for colorectal cancers in our department. We reviewed 22 (2.6%) consecutive patients who presented with perforation at one institution.

Results. Fifteen (69%) patients underwent potentially curative resection. The overall operative morbidity and mortality rates were 50% and 9%. The overall 5-year survival rate was 17.4%, although by excluding patients who either had stage IV disease at diagnosis or who died during or soon after surgery (n = 7), the 5-year survival rate increased to 32% (n = 15). Furthermore, the 5-year survival rate of patients who underwent a potentially curative resection (36.9%) was significantly better than that of those who underwent a noncurative resection (0%, P = 0.0093).

Conclusions. Perforating colorectal cancers are associated with high postoperative mortality and poor long-term survival. However, the intensive management of radical lymph node dissection and surgical resection are recommended to improve the long-term prognosis.

Key words Curative surgery · Perforated colorectal cancer

Introduction

Colorectal cancer remains the third most common type of cancer in Japan. Each year, approximately 94 000 new cases are diagnosed and almost 39 000 people die of the disease.[1] The overall survival rate is poor: many patients have locally advanced or metastatic disease at the time of presentation, and only half of those who undergo a potentially curative resection survive for 5 years.

Perforation, obstruction, bleeding, and invasion of the adjoining organs are the major complications associated with locally advanced colorectal cancer. Perforation has been reported to occur in 3%–9% of colorectal cancers, and is associated with a high postoperative mortality and a poor long-term outcome.[2–7] This presentation adds the morbidity and mortality of sepsis and peritonitis to that already associated with emergency surgery for colorectal cancer, and appears to reduce the 5-year survival by 20%.[2]

The aim of the present study was to elucidate the postoperative morbidity and long-term outcome of patients presenting with perforation in the setting of colorectal cancer, and to search for the indicators of prognosis and possible causes of death. To evaluate the risk of this complication, we compared the data of patients with perforated colorectal cancers with those of patients with uncomplicated colorectal cancers.

Patients and Methods

Between 1996 and 2004, 22 patients underwent emergency surgery for perforated colorectal cancer, among a total of 848 patients with colorectal cancer treated for colorectal cancer during the same period at one institution. Their clinical data, prospectively stored in a database, were retrieved and analyzed for this study. The tumors were classified according to site as follows: lesions of the cecum, ascending colon, and hepatic flexure were classified as right-sided lesions, whereas lesions of the transverse colon, splenic flexure, and descending colon were classified as left-sided colon lesions. Tumor staging was assessed by the TNM and Dukes' classification based on histological examinations

Reprint requests to: K. Shibahara, Department of Surgery, Munakata Medical Association Hospital, 5-5-3 Taguma, Munakata, Fukuoka 811-3431, Japan
Received: May 5, 2009 / Accepted: July 7, 2009

of resected specimens and the findings of radiological examinations, such as chest X-ray and computed tomography.

Patients were deemed to have undergone a curative resection if the surgeon considered that there was no macroscopic residual tumor once the resection was completed. Patients with distant metastases who underwent resection and patients in whom inadequate local clearance was achieved were deemed to have undergone a palliative resection. The curability and type of lymph node dissection were described according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum, and Anus.[8]

Follow-up examination after discharge was conducted either at our hospital or at a private physician's office every 3 months for 2 years, every 6 months for the next 3 years, and annually thereafter. Concurrent follow-up was also performed in our tumor registry. Proportions were compared using the chi-squared test for large samples and Fisher's exact probability test for small samples. Continuous data were compared using a nonparametric Wilcoxon test. Differences were considered significant at a P value of less than 0.05. Survival curves were analyzed by the Kaplan–Meier method and tests of significance were done using the log-rank test.

Results

Of the 848 patients with colorectal cancer, retrospectively analyzed, 22 (2.6%) presented with pathologically proved perforated lesions. The pathological findings confirming perforation included ulceration and splitting of all layers of the bowel wall, necrosis or chronic inflammatory reaction, and abscess formation around the perforation site. The incidence of the various clinicopathological factors are shown in Table 1. The primary tumor was most often located on the left side of the colon ($n = 21$, 96%), especially the sigmoid colon and rectum ($n = 16$, 73%). The perforation was located at the tumor site in 20 (91%) patients, and proximal to the obstructed primary lesion in the other 2 (9%) patients. We were able to diagnose the colorectal cancers preoperatively by computed tomography in only 12 (54.5%) patients.

All 22 patients underwent surgery; however, the resectability rate was 86%, as 3 patients underwent only a palliative colostomy. Fourteen patients (64%) underwent a potentially curative resection and 6 (27%) had metastatic disease at the time of surgery. Distant metastases were found in the liver in 3 patients, the peritoneum in 3, and the lung in 1. One of these six patients underwent a relatively curative resection combined with an adenexectomy for an ovarian metastasis. Seven of the 13 patients who underwent potentially curative surgery received oral adjuvant chemotherapy. The other patients did not receive adjuvant chemotherapy because of either complications or their age. The operative morbidity and mortality rates are shown in Table 2. Septic shock accounted for two early deaths. The patients with perforated colorectal cancer had significantly higher postoperative morbidity and mortality rates than the patients with uncomplicated cancers.

Table 1. Patients and tumor characteristics

Characteristic	No. of patients (%)
Total	22 (100)
Sex	
Male	10 (45)
Female	12 (55)
Median (range) age (years)	71.8 (34–92)
Cancer site	
Right	1 (4)
Left	5 (23)
Sigma	9 (41)
Rectum	7 (32)
Median (range) tumor diameter (cm)	6.6 (2–18)
TNM stage	
I	0 (0)
II	11 (50)
III	5 (23)
IV	6 (27)
Dukes' stage	
A	1 (5)
B	13 (59)
C	2 (9)
D	6 (27)
Lymph node dissection	
D0	3 (14)
D1	7 (32)
D2	10 (4)
D3	2 (9)
Curability	
A	14 (64)
B	1 (5)
C	7 (31)
Surgical procedure	
Hartmann's procedure	11 (50)
Primary resection	
With anastomosis	6 (27)
With a colostomy	2 (9)
Without a colostomy	4 (18)
Without anastomosis	2 (9)
Colostomy alone	3 (14)

The mean follow-up was 18.9 months (range, 1–133 months). At the time of writing, four patients were alive without recurrence, three were alive with liver metastasis, nine had died of relapse of colorectal cancer (as liver metastasis in four, peritoneal dissemination in three, brain metastasis in one, and bone metastasis in one), and four had died of unrelated causes. The survival data of our study (Fig. 1) revealed an overall 5-year survival rate of 17.4% for the 22 cases; however, the exclusion of seven patients with either stage IV disease at the time of perforation, or who died during or soon after their

Table 2. Postoperative complications and mortality in perforated and uncomplicated cancers

	No. (%)		
	Perforating cancer ($n = 22$)	Uncomplicated cancer ($n = 826$)	P value
Mortality	2 (9)	8 (1)	0.0255
Complications			
Total	11 (50)	193 (23.4)	0.0076
Anastomotic leak	2 (9.1)	22 (2.7)	0.1257
Abdominal abscess	3 (13.6)	3 (0.4)	0.0003
Wound infection	6 (27.3)	50 (6)	0.0021
Sepsis	2 (9.1)	3 (0.4)	0.0061
Respiratory disturbance	3 (13.6)	19 (2.3)	0.0170
Cardiovascular disturbance	0 (0)	13 (1.6)	>0.05
Others	0 (0)	83 (10)	>0.05

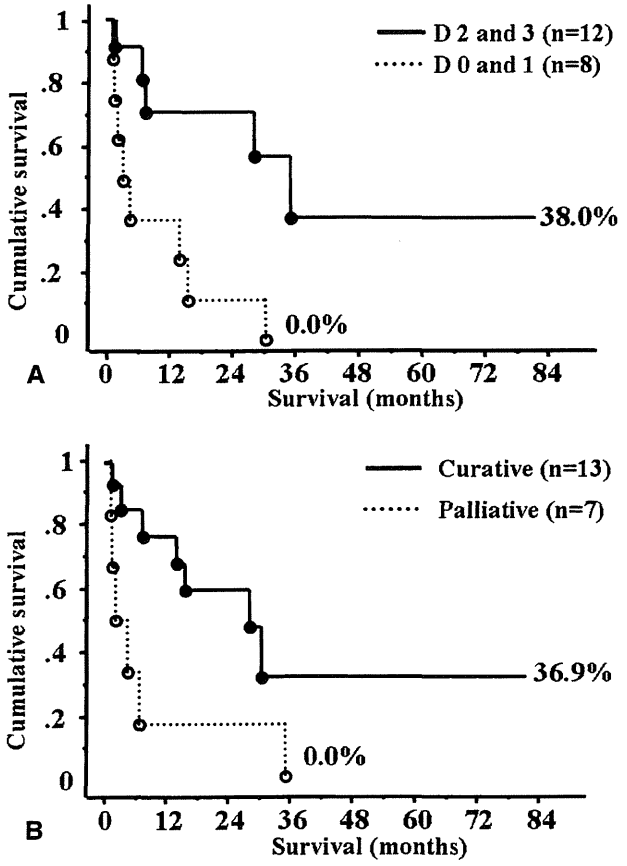

Fig. 1. **A** Survival curves for patients who underwent potentially radical D2 or D3 lymph node dissection ($n = 12$), or nonradical D0 or D1 lymph node dissection ($n = 8$) ($P = 0.0045$, log-rank test). **B** Survival curves for patients who underwent either a potentially curative resection ($n = 13$) or a palliative resection ($n = 7$) ($P = 0.0108$, log-rank test)

operation, resulted in 15 patients who had a mean survival of 28 months and a 5-year survival rate of 32%. Excluding postoperative mortality, the overall survival was 38% following potentially radical lymph node dissection (so-called D2 or D3), as compared with 0% in patients who underwent nonradical lymph node dissection (so-called D0 or D1), respectively ($P = 0.0045$). The patients who underwent a potentially curative resection had a significantly better crude 5-year survival rate than those who underwent palliative resection (36.9% vs 0%; $P = 0.0108$).

Discussion

Perforation has been reported to occur in 3%–9% of colorectal cancers,[2,3,9,10] although the incidence in our study was slightly lower, at 2.6%. Perforated colorectal cancer is generally associated with a low survival rate and high postoperative mortality.[2–4,6] Its outcome is worse than that of other complicated presentations including obstruction and bleeding,[6] the reasons for which are multifactorial. The survival and outcome of these patients depends on their general condition, the severity of sepsis, if there is locally advanced malignancy, and the presence of metastatic disease at the time of perforation.[4,9] The operative mortality was 9% in our series, which compares favorably to previously reported mortality rates of 30%–43%.[2,4,5,9] It is not surprising that the postoperative morbidity and mortality rates are higher in these patients than in those who undergo elective surgery for colorectal cancer. Many of these patients are elderly, with a mean age of 71.8 years in our study, and therefore likely to suffer from cardiovascular or respiratory dysfunction before their acute admission.[11] Moreover, advanced age tends to delay diagnosis, and peritonitis may develop rapidly in this population. Under these circumstances, the timing of the diagnosis may determine survival.

Traditionally, Hartmann's procedure, or resection and double-barrel colostomy, was the surgical procedure of choice, but now one-staged procedures are often performed with a significant survival advantage.[3,5,10] Most (96%) of the patients in this series had

left colon lesions and 15 (68%) underwent Hartmann's procedure, or resection and colostomy, while only four underwent resection and primary anastomosis. A curative resection was possible in 69% of the patients, which compares favorably with historical rates of resection (60%–92%).[12,13] Our rationale for attempting potentially curative resection was supported by results reported by others.[10,14] These researchers found that the type of surgical treatment played an important role in prognosis. Their retrospective review of patients presenting with obstructing or perforated cancers revealed a significant decrease in hospital mortality and improvement in both 3-year and 5-year survival for those patients who underwent a potentially curative surgery versus a staged drainage procedure.

The crude 5-year survival for patients with perforation of colorectal cancer has been reported to range from 7% to 44%.[3,9] Although the overall 5-year survival rate in this study population was 17.4%, when adjusted to exclude operative mortality and patients with metastatic disease at the onset, it increased to 32%.

The en bloc curative operation, potentially including radical lymph node dissection, or so-called D2 or D3, may be warranted, in accordance with other reports.[10,14,15] In the present series, patients with stage IV disease at diagnosis and those who died within the 30-day postoperative period were excluded from the total 15 patients who underwent potentially curative resection, yielding 13 patients. This group of 13 patients was found to have a mean survival of 30 months and a 5-year survival rate of 36.9%; significantly better than that of those who underwent a noncurative resection (0%, $P = 0.0108$). This 5-year survival rate of 36.9% compared favorably with data on patients with colorectal cancer reported from Japan and with the reported 13.2% 5-year survival rate of patients with stage IV disease.[8]

The results of the present study show that patients presenting with perforated cancers tend to have advanced disease, a low curative resection rate, a high postoperative mortality rate, and poor overall survival. Hence, intensive treatment, including sepsis management and surgery, is usually warranted. Adjuvant chemotherapy is effective for advanced colorectal cancer[5] and may therefore be routinely recommended for patients with perforated colorectal cancer.

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J Pharmacol Sci **112**, 473 – 476 (2010)

Journal of Pharmacological Sciences

Short Communication

Mexiletine Reverses Oxaliplatin-Induced Neuropathic Pain in Rats

Nobuaki Egashira[1,*], Shingo Hirakawa[1], Takehiro Kawashiri[1], Takahisa Yano[1], Hiroaki Ikesue[1], and Ryozo Oishi[1]

[1]*Department of Pharmacy, Kyushu University Hospital, Fukuoka 812-8582, Japan*

Received January 14, 2010; Accepted February 15, 2010

Abstract. Oxaliplatin is a platinum-based chemotherapy drug characterized by the development of acute and chronic peripheral neuropathies. Mexiletine, an orally available Na^+-channel blocker, has widely been used in patients with chronic painful diabetic neuropathy. In the present study, we examined the effect of mexiletine on oxaliplatin-induced neuropathic pain in rats. Mexiletine (100, but not 10 and 30, mg/kg, p.o.) completely reversed both mechanical allodynia and cold hyperalgesia induced by oxaliplatin (4 mg/kg, i.p., twice a week). Lidocaine (30, but not 3 and 10, mg/kg, i.p.) also significantly relieved both pain behaviors. These results suggest that mexiletine may be effective in relieving the oxaliplatin-induced neuropathic pain clinically.

Keywords: mexiletine, oxaliplatin, neuropathic pain

Oxaliplatin, a third-generation platinum-based chemotherapy drug, is a key drug in the treatment of colorectal cancer. Unlike other platinum compounds, oxaliplatin induces an acute painful neuropathy, which appears soon after administration (1). The patients suffer from extremity and perioral paresthesias and in particular from severe cold hypersensitivity. After multiple cycles the patients develop a clinically different peripheral neuropathy that is characterized by a sensory axonal nerve damage closely resembling that induced by cisplatin. This chronic neuropathy can become very disabling and is, in fact, often a dose-limiting toxicity. For this reason, peripheral neuropathy associated with the administration of oxaliplatin is a major clinical problem in chemotherapy.

Mexiletine, an orally available Na^+-channel blocker, has been reported to be effective on chronic painful diabetic neuropathy in clinical trial (2), and it is prescribed for treating patients with these symptoms. In animal models, acute administration of mexiletine has been reported to relieve the mechanical allodynia in rats treated with vincristine, a chemotherapeutic agent, and streptozotocin-induced diabetic rats (3, 4). No experimental study, however, has been conducted to date to determine the effect of mexiletine on pain behavior in a rat model of oxaliplatin-induced neuropathy. In the present study, we examined the effect of mexiletine on the oxaliplatin-induced mechanical allodynia and cold hyperalgesia after the development of neuropathy in rats.

Male Sprague-Dawley rats weighing 200 – 250 g (Kyudo Co., Saga) were used in the present study. Rats were housed in groups of four to five per cage, with lights on from 08:00 to 20:00 h. Animals had free access to food and water in their home cages. All experiments were approved by the Experimental Animal Care and Use Committee of Kyushu University according to the National Institutes of Health guidelines, and we followed IASP Committee for Research and Ethical Issues guidelines for animal research (5).

Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo). Mexiletine hydrochloride was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Lidocaine (Xylocaine® 2% for intravenous injection) was obtained from Astra Zeneca K.K. (Osaka). Oxaliplatin was dissolved in 5% glucose solution. The vehicle-treated rats were injected with 5% glucose solution. Oxaliplatin (4 mg/kg) or vehicle was injected intraperitoneally (i.p.) twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22, and 23). Mexiletine was dissolved in sterile water and administered orally. Lidocaine was dissolved in saline and administered i.p. The doses of these drugs were chosen based on previous reports (3, 4, 6). Behavioral tests were performed blindly with respect to drug administration.

The mechanical allodynia was assessed by the von Frey test. Rats were placed in a clear plastic box

*Corresponding author. n-egashi@pharm.med.kyushu-u.ac.jp
Published online in J-STAGE on March 20, 2010 (in advance)
doi: 10.1254/jphs.10012SC

(20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. von Frey filaments (The Touch Test Sensory Evaluator Set; Linton Instrumentation, Norfolk, UK) of 1 – 15 g bending force were applied to the midplantar skin of each hind paw with each application held for 6 s. Fifty percent paw withdrawal thresholds were determined by up-down methods (7).

The cold hyperalgesia was assessed by the acetone test described by Flatters and Bennett (8). Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. A 50-μL aliquot of acetone (Wako Pure Chemical, Ltd., Osaka) was sprayed onto the plantar skin of each hind paw three times with a Micro Sprayer® (Penn Century Inc., Philadelphia, PA, USA), and the number of withdrawal responses was counted for 40 s from the start of the acetone spray.

We confirmed the incidence of mechanical allodynia and cold hyperalgesia on Days 24 and 3, respectively. We carried out the drug evaluation on the next day. In the case of mexiletine, the von Frey and acetone tests were performed immediately before (0 min) and at 60, 120, and 180 min after administration. In the case of lidocaine, the von Frey and acetone tests were performed immediately before (0 min) and at 30, 60, and 120 min after administration.

Values were expressed as the mean ± S.E.M. The values were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey-Kramer's post-hoc test (StatView; Abacus Concepts, Berkeley, CA, USA) to determine differences among the groups. A probability level of $P < 0.05$ was accepted as statistically significant.

Oxaliplatin (4 mg/kg, i.p.) significantly reduced the 50% paw withdrawal threshold compared with the vehicle in the von Frey test on Day 24 ($P < 0.01$, Figs. 1A and 2A). Oxaliplatin at the same dose significantly increased the number of withdrawal responses compared with vehicle in the acetone test on Day 3 ($P < 0.01$, Figs. 1B and 2B). The incidence of mechanical allodynia and cold hyperalgesia was 92% and 81%, respectively. Acute administration of mexiletine (100 mg/kg, p.o.) completely reversed the reduction of 50% paw withdrawal threshold by oxaliplatin at 60 min after administration in the von Frey test ($P < 0.01$, Fig. 1A). Moreover, mexiletine (100 mg/kg, p.o.) completely reversed the increase of number of withdrawal responses by oxaliplatin at 60 and 120 min after administration in the acetone test ($P < 0.05$, Fig. 1B). These effects of mexiletine disappeared by 180 min after administration. Similarly, acute administration of lidocaine (30 mg/kg, i.p.) significantly inhibited the reduction of 50% paw withdrawal threshold by oxaliplatin at 30 min after administration in the von Frey test ($P < 0.05$, Fig. 2A). Moreover, lidocaine (3, 10, and 30 mg/kg, i.p.) significantly inhibited the increase of number of withdrawal responses by oxaliplatin at 30 min after administration in the acetone test ($P < 0.01$, Fig. 2B). These effects of lidocaine had disappeared by 120 min after administration. In addition, mexiletine (100 mg/kg, p.o.) and lidocaine (30 mg/kg, i.p.) had no effect on the 50% paw withdrawal threshold in the von Frey test and the number of withdrawal responses in the acetone test in intact rats (data not shown).

Our data in this study revealed that acute administration of mexiletine completely reversed both mechanical

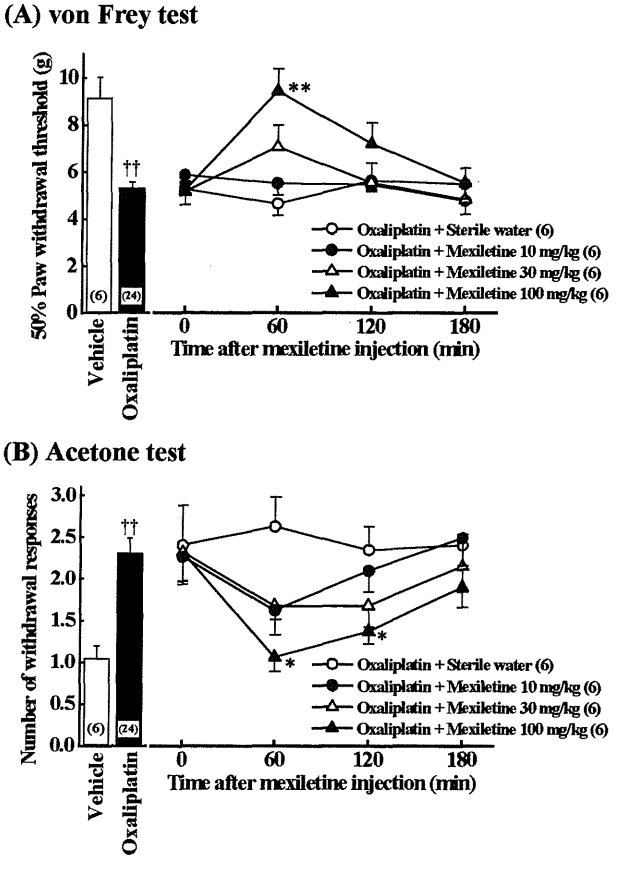

Fig. 1. Effect of mexiletine on mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test (B) in oxaliplatin-treated rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22, and 23). We confirmed the incidence of mechanical allodynia and cold hyperalgesia on Days 24 and 3, respectively. We carried out the drug evaluation on the next day. Mexiletine was administered orally. The von Frey and acetone tests were performed immediately before (0 min) and at 60, 120, and 180 min after administration. Number of animals is shown in parenthesis. Values are expressed as the mean ± S.E.M. $^{\dagger\dagger}P < 0.01$, compared with vehicle; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with oxaliplatin alone.

allodynia and cold hyperalgesia induced by oxaliplatin. Mexiletine has widely been used in the treatment of chronic painful diabetic neuropathy. It has also been reported that mexiletine produced no major adverse events and was superior to placebo to relieve neuropathic pain in controlled clinical trials (9). Taken together, the present results suggest that mexiletine is useful as a therapeutic drug for oxaliplatin-induced neuropathic pain if it is used with caution as needs arise.

Similarly, lidocaine, another Na^+-channel blocker, significantly relieved both pain behaviors. Ling and colleagues (10) have reported that single intravenous administration of lidocaine relieved the oxaliplatin-induced cold allodynia in rats. Our finding is essentially consistent with the previous finding. Moreover, we found that mexiletine and lidocaine at the effective dose had no effect on pain behavior in intact rats. Therefore, the ameliorative effects of mexiletine and lidocaine were not attributable to non-specific sedative effects or a deficit of motor function. These findings suggest that the reduced pain behavior by Na^+-channel blockers reflects a therapeutic effect on oxaliplatin-induced neuropathic pain. Asano et al. (11) reported that mexiletine at the dose of 20 mg/kg did not affect pain-related responses in normal mice. They also indicated that activation of the descending β-endorphinergic system is involved in the antinociceptive effect of mexiletine. The β-endorphinergic system is generally accepted as an antinociceptive system, which selectively has antinociceptive effect on painful conditions. In the in vitro studies, application of oxaliplatin to dorsal root ganglion (DRG) neurons resulted in an increase of the Na^+ current (12). Interestingly, the effect of oxaliplatin is antagonized by the Na^+-channel blocker carbamazepine (12). Therefore, mexiletine and lidocaine exhibit effective relief on the oxaliplatin-induced neuropathic pain, but may be ineffective in reducing pain-related behaviors in intact rats.

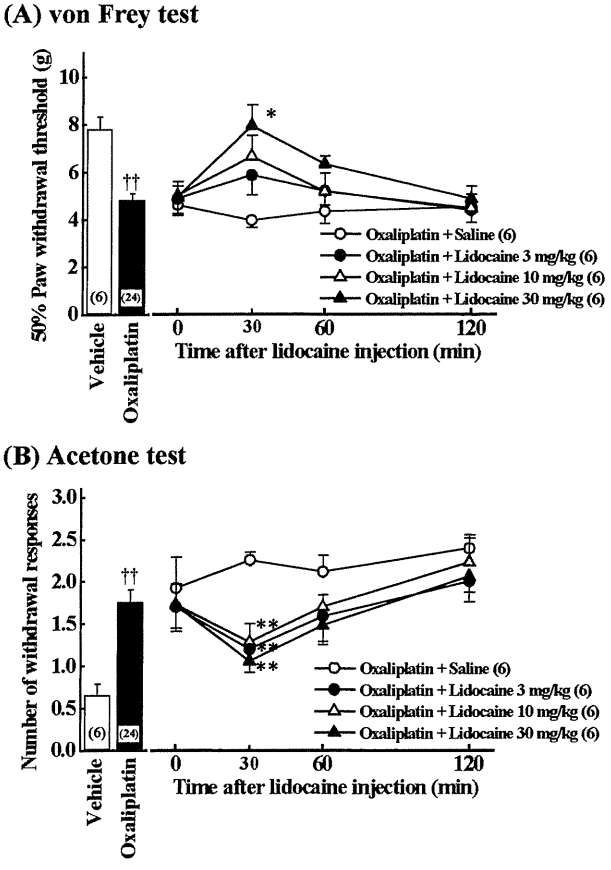

Fig. 2. Effect of lidocaine on mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test (B) in oxaliplatin-treated rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22, and 23). We confirmed the incidence of mechanical allodynia and cold hyperalgesia on Days 24 and 3, respectively. We carried out the drug evaluation on the next day. Lidocaine was administered i.p. The von Frey and acetone tests were performed immediately before (0 min) and at 30, 60, and 120 min after administration. Number of animals is shown in parenthesis. Values are expressed as the mean ± S.E.M. $^{\dagger\dagger}P < 0.01$, compared with vehicle; $^*P < 0.05$, $^{**}P < 0.01$, compared with oxaliplatin alone.

In the present study, mexiletine reversed mechanical allodynia and cold hyperalgesia to the same degree. Lidocaine also relieved both pain-related behaviors. Recently, we demonstrated that oxalate and platinum metabolite are involved in the cold hyperalgesia and mechanical allodynia, respectively (6). Oxalate alters voltage-gated Na^+ channels (13) and its effect may be involved in the cold hyperalgesia. On the other hand, the mechanical allodynia may be due to the peripheral nerve injury by platinum metabolite. The change in the expression of Na^+ channels is observed after peripheral nerve injury of the rat DRG neurons (14). Taken together with these findings, the present results suggest that mexiletine and lidocaine may reverse the mechanical allodynia and cold hyperalgesia by inhibiting the hyperexcitability of Na^+ channels.

In conclusion, the study presented here demonstrates, for the first time, that acute administration of mexiletine reverses both mechanical allodynia and cold hyperalgesia induced by oxaliplatin in rats.

Acknowledgment

Part of this study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (No. 21590285).

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Mihara et al. Molecular Pain 2011, 7:8
http://www.molecularpain.com/content/7/1/8

MOLECULAR PAIN

RESEARCH Open Access

Involvement of spinal NR2B-containing NMDA receptors in oxaliplatin-induced mechanical allodynia in rats

Yuki Mihara, Nobuaki Egashira*, Hikaru Sada, Takehiro Kawashiri, Soichiro Ushio, Takahisa Yano, Hiroaki Ikesue, Ryozo Oishi

Abstract

Background: Oxaliplatin is a platinum-based chemotherapy drug characterized by the development of acute and chronic peripheral neuropathies. The chronic neuropathy is a dose-limiting toxicity. We previously reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats. In the present study, we investigated the involvement of NR2B-containing N-methyl-D-aspartate (NMDA) receptors in oxaliplatin-induced mechanical allodynia in rats.

Results: Repeated administration of oxaliplatin (4 mg/kg, i.p., twice a week) caused mechanical allodynia in the fourth week, which was reversed by intrathecal injection of MK-801 (10 nmol) and memantine (1 μmol), NMDA receptor antagonists. Similarly, selective NR2B antagonists Ro25-6981 (300 nmol, i.t.) and ifenprodil (50 mg/kg, p.o.) significantly attenuated the oxaliplatin-induced pain behavior. In addition, the expression of NR2B protein and mRNA in the rat spinal cord was increased by oxaliplatin on Day 25 (late phase) but not on Day 5 (early phase). Moreover, we examined the involvement of nitric oxide synthase (NOS) as a downstream target of NMDA receptor. L-NAME, a non-selective NOS inhibitor, and 7-nitroindazole, a neuronal NOS (nNOS) inhibitor, significantly suppressed the oxaliplatin-induced pain behavior. The intensity of NADPH diaphorase staining, a histochemical marker for NOS, in the superficial layer of spinal dorsal horn was obviously increased by oxaliplatin, and this increased intensity was reversed by intrathecal injection of Ro25-6981.

Conclusion: These results indicated that spinal NR2B-containing NMDA receptors are involved in the oxaliplatin-induced mechanical allodynia.

Background

Glutamate is a major excitatory transmitter in the spinal cord and N-methyl-D-aspartate (NMDA) receptors are known to be involved in the painful neuropathy [1,2]. The NMDA receptor antagonist inhibits the pain hypersensitivity in chronic constriction injury (CCI) model. Moreover, the expression of spinal NR2B-containing NMDA receptors is increased with the pain hypersensitivity induced by CCI or chronic compression of the dorsal root ganglia (CCD) [3-6]. Selective NR2B antagonists inhibit mechanical allodynia without causing motor dysfunction in CCI, CCD and spinal nerve-ligated (SNL) neuropathic models [5-8]. In addition, the NR2B subunits are localized to the superficial dorsal horn of the spinal cord [7,9], suggesting a possible involvement in pain transmission. Thus, the NR2B-containing NMDA receptors may play an important role in the neuropathic pain.

Nitric oxide synthase (NOS) as a downstream target of NMDA receptor also contributes greatly to the incidence of neuropathic pain. In the rat CCI model of neuropathic pain, intrathecal injection of non-selective NOS inhibitor L-N^G-nitro-arginine methyl ester (L-NAME) reverses the persistent thermal hyperalgesia [10]. Furthermore, a close correlation between neuronal NOS (nNOS) and neuropathic pain has been documented in CCI model [11].

Oxaliplatin, a third-generation platinum-based chemotherapy drug, has widely been used as a key drug in

* Correspondence: n-egashi@pharm.med.kyushu-u.ac.jp
Department of Pharmacy, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

the treatment of colorectal cancer. However, oxaliplatin causes severe acute and chronic peripheral neuropathies. The acute neuropathy includes acral paresthesias and dysesthesia triggered or enhanced by exposure to cold, and it appears soon after administration [12]. After multiple cycles the patients develop the chronic neuropathy that is characterized by a sensory and motor dysfunction. This chronic neuropathy is a dose-limiting toxicity and a major clinical problem in oxaliplatin chemotherapy [13].

Recently, we reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats [14]. Oxaliplatin is metabolized to oxalate and dichloro(1,2-diaminocyclohexane)platinum [Pt(dach)Cl_2] [15]. We demonstrated that oxalate and platinum metabolite are involved in the cold hyperalgesia and mechanical allodynia, respectively [14]. Oxalate alters voltage-gated Na^+ channels [16] and its effect may be involved in the cold hyperalgesia. On the other hand, the mechanical allodynia may be due to the peripheral nerve injury by platinum metabolite. However, whether the NR2B-containing NMDA receptors are involved still remains largely unclear. In the present study, we investigated the involvement of NR2B-containing NMDA receptors in the oxaliplatin-induced mechanical allodynia in rats.

Results

Effects of NMDA receptor antagonists on oxaliplatin-induced mechanical allodynia

Oxaliplatin (4 mg/kg, i.p., twice a week for 4 weeks) significantly reduced the paw withdrawal thresholds compared with the vehicle in the von Frey test on Day 24 ($P < 0.01$, Figure 1). Acute administration of a NMDA receptor antagonist MK-801 (10 nmol, i.t.) completely reversed the reduction of paw withdrawal threshold by oxaliplatin at 30 min after administration ($P < 0.05$, Figure 1A). Similarly, acute administration of another NMDA receptor antagonist memantine (1 μmol, i.t.) completely reversed the reduction of paw withdrawal threshold by oxaliplatin at 30 min after administration ($P < 0.05$, Figure 1B). These effects of MK-801 and memantine disappeared by 120 min after administration. In addition, MK-801 (10 nmol, i.t.) and memantine (1 μmol, i.t.) had no effect on the paw withdrawal thresholds in intact rats (data not shown).

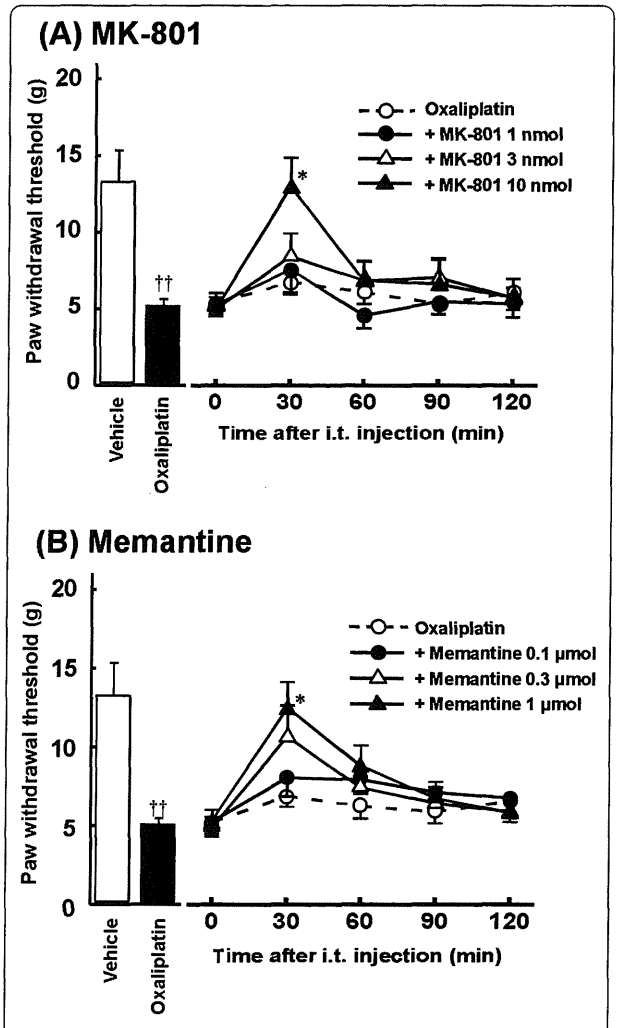

Figure 1 Effects of MK-801 (A) and memantine (B) on oxaliplatin-induced mechanical allodynia in the von Frey test. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22 and 23). We confirmed the incidence of mechanical allodynia on Day 24. We carried out the drug evaluation on the next day. MK-801 (1-10 nmol/body) and memantine (0.1-1 μmol/body) were administered intrathecally. The von Frey test was performed immediately before (0 min) and at 30, 60, 90 and 120 min after administration. Values are expressed as mean ± SEM of 6-10 animals. $^{††}P < 0.01$ compared with vehicle, $^{*}P < 0.05$ compared with oxaliplatin alone.

Effects of NR2B antagonists on oxaliplatin-induced mechanical allodynia

Acute administration of a selective NR2B antagonist Ro 25-6981 (300 nmol, i.t.) significantly inhibited the reduction of paw withdrawal threshold by oxaliplatin at 30 and 60 min after administration ($P < 0.01$: 30 min, $P < 0.05$: 60 min, Figure 2A). Similarly, acute administration of another NR2B antagonist ifenprodil (50 mg/kg, p.o.) significantly inhibited the reduction of paw withdrawal threshold by oxaliplatin at 30 and 60 min after administration ($P < 0.01$: 30 min, $P < 0.05$: 60 min, Figure 2B). These effects of Ro 25-6981 and ifenprodil disappeared by 120 min after administration. In addition, Ro 25-6981 (300 nmol, i.t.) and ifenprodil (50 mg/kg, p.o.) had no effect on the paw withdrawal thresholds in intact rats (data not shown).

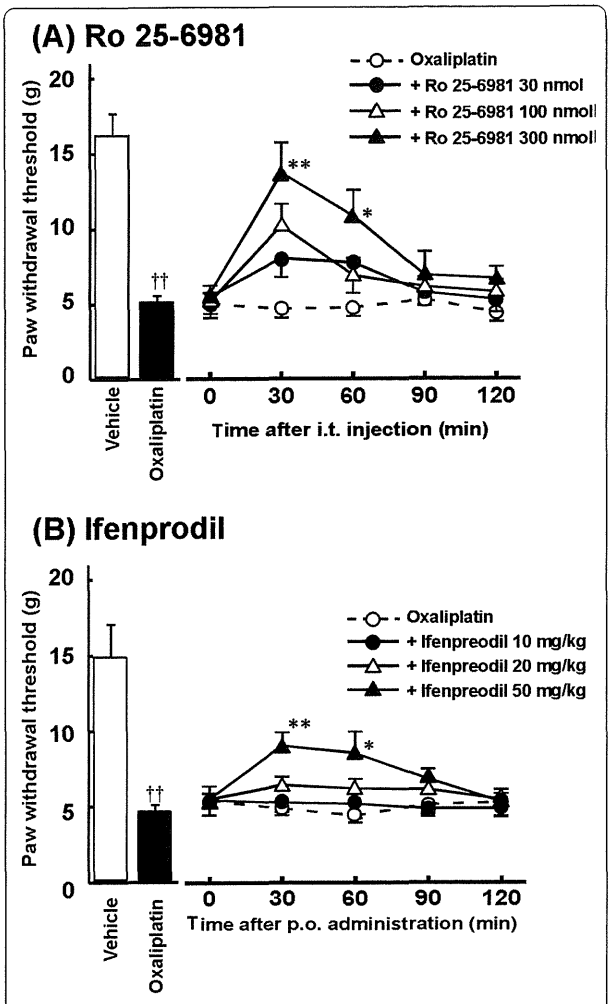

Figure 2 Effects of Ro 25-6981 (A) and ifenprodil (B) on oxaliplatin-induced mechanical allodynia in the von Frey test. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22 and 23). We confirmed the incidence of mechanical allodynia on Day 24. We carried out the drug evaluation on the next day. Ro 25-6981 (30-300 nmol/body) was administered intrathecally. Ifenprodil (10-50 mg/kg) was administered orally. The von Frey test was performed immediately before (0 min) and at 30, 60, 90 and 120 min after administration. Values are expressed as mean ± SEM of 6-7 animals. $^{\dagger\dagger}P < 0.01$ compared with vehicle, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with oxaliplatin alone.

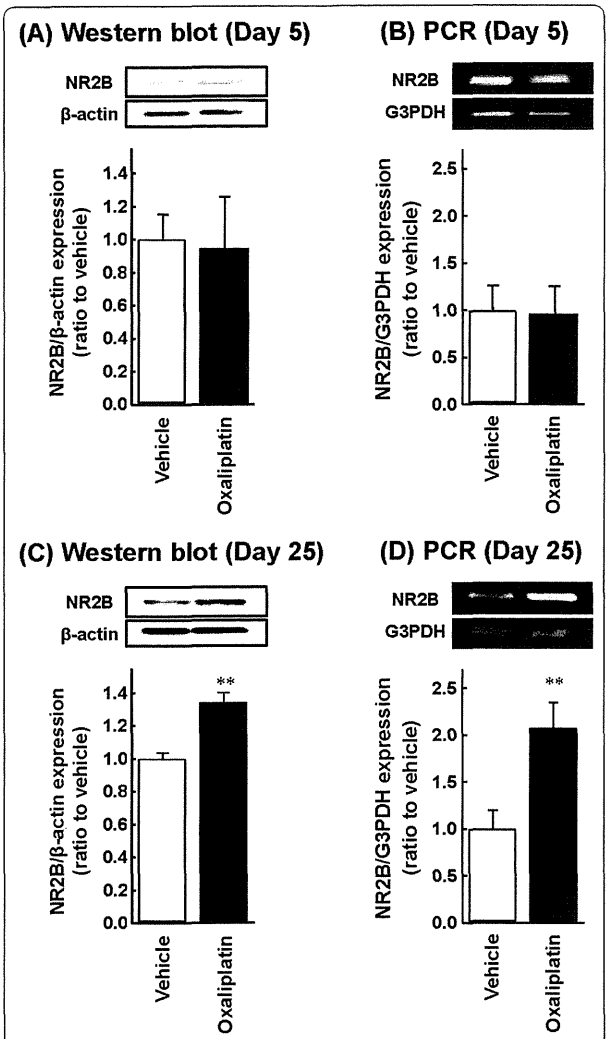

Figure 3 Changes in the NR2B protein (A, C) and mRNA (B, D) levels in the spinal cord in oxaliplatin-treated rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 1 (Days 1 and 2) or 4 weeks (Days 1, 2, 8, 9, 15, 16, 22 and 23). Western blot and PCR were performed to measure NR2B expression at the time points of Days 5 (A, B) and 25 (C, D) in the L4-6 spinal cord. Values are expressed as mean ± SEM of 6-9 animals. $^{**}P < 0.01$ compared with vehicle.

Changes of NR2B protein and mRNA in the spinal cord in oxaliplatin-treated rats

NR2B expression was examined by Western blot and polymerase chain reaction (PCR) analysis on homogenates of the spinal cord from rats. The results of Western blot and PCR showed that NR2B protein and mRNA levels in the spinal cord of oxaliplatin-treated rats significantly increased compared with that of vehicle-treated rats on Day 25 ($P < 0.01$, Figures 3C, D). On the other hand, oxaliplatin caused no change in NR2B protein and mRNA levels in the spinal cord on Day 5 (Figures 3A, B).

Effects of NOS inhibitors on oxaliplatin-induced mechanical allodynia

Acute administration of a non-selective NOS inhibitor L-NAME (300 μg, i.t.) completely reversed the reduction of paw withdrawal threshold by oxaliplatin at 30 min after administration ($P < 0.05$, Figure 4A). Similarly, acute administration of an nNOS inhibitor

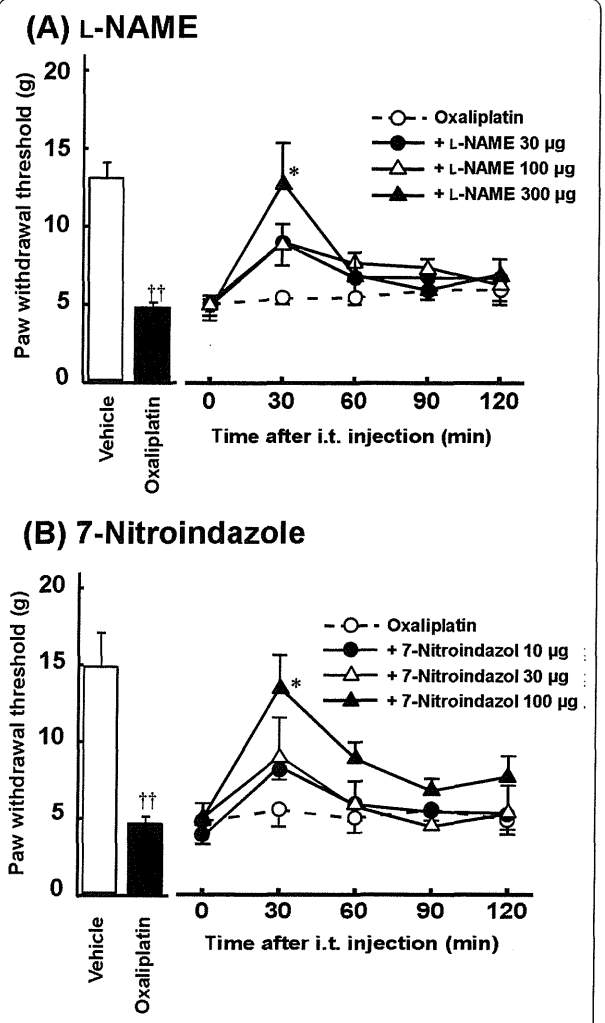

Figure 4 Effects of L-NAME (A) and 7-nitroindazole (B) on oxaliplatin-induced mechanical allodynia in the von Frey test. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22 and 23). We confirmed the incidence of mechanical allodynia on Day 24. We carried out the drug evaluation on the next day. L-NAME (30-300 μg/body) and 7-nitroindazole (10-100 μg/body) were administered intrathecally. The von Frey test was performed immediately before (0 min) and at 30, 60, 90 and 120 min after administration. Values are expressed as mean ± SEM of 6-10 animals. $^{\dagger\dagger}P < 0.01$ compared with vehicle, $^{*}P < 0.05$ compared with oxaliplatin alone.

7-nitroindazole (100 μg, i.t.) significantly inhibited the reduction of paw withdrawal threshold by oxaliplatin at 30 min after administration ($P < 0.05$, Figure 4B). These effects of L-NAME and 7-nitroindazole disappeared by 120 min after administration. In addition, L-NAME (300 μg, i.t.) and 7-nitroindazol (100 μg, i.t.) had no effect on the paw withdrawal thresholds in intact rats (data not shown).

Change of NOS activity in the spinal cord of oxaliplatin-treated rats

To evaluate change of NOS activity in oxaliplatin-induced mechanical allodynia, we carried out the NADPH diaphorase staining, a histochemical marker for NOS, in rat spinal cord sections. The results of NADPH diaphorase histochemistry revealed that the intensity of NADPH diaphorase staining (blue staining) obviously increased in the superficial layer of spinal dorsal horn in oxaliplatin-treated rats on Day 25 (Figure 5). Moreover, this increased intensity was reversed by intrathecal injection of Ro 25-6981 (300 nmol).

Discussion

In this study, NMDA receptor antagonists completely reverse the oxaliplatin-induced mechanical allodynia when administered after the development of neuropathy. Similarly, selective NR2B antagonists significantly inhibited the oxaliplatin-induced mechanical allodynia. Moreover, the expression of NR2B protein and mRNA in the spinal cord increased in the oxaliplatin-treated rats on Day 25 (late phase) but not on Day 5 (early phase). Oxaliplatin (4 mg/kg, i.p., twice a week) induces cold hyperalgesia in the early phase and mechanical allodynia in the late phase [14]. These findings suggest that the up-regulation of spinal NR2B-containing NMDA receptors is involved in the incidence of mechanical allodynia by repeated administration of oxaliplatin.

To investigate whether spinal cord NOS as the downstream target of NMDA receptor contributes to the incidence of mechanical allodynia, we examined the effects of NOS inhibitors on the oxaliplatin-induced mechanical allodynia. Intrathecal injection of L-NAME, a non-selective NOS inhibitor, and 7-nitroindazole, a selective nNOS inhibitor, inhibited the pain behavior, suggesting that NOS especially nNOS is involved in the oxaliplatin-

Figure 5 Typical photomicrographs representing NOS histochemistry staining of neurons in the spinal cord. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22 and 23). On Day 25, Ro 25-6981 (300 nmol/body) was administered intrathecally, and the L4-6 spinal cord was removed 30 min after administration. NADPH diaphorase staining was performed to confirm NOS activity. The blue staining shows NADPH diaphorase staining. Spinal slices (30 μm) were prepared from rats treated with vehicle (A), oxaliplatin alone (B), and oxaliplatin and Ro 25-6981 (C). Scale bar, 200 μm.

induced mechanical allodynia. This is further supported by the finding that the intensity of NADPH diaphorase staining in the rat spinal dorsal horn was increased by repeated administration of oxaliplatin, and that this increased intensity was reversed by intrathecal injection of Ro25-6981, which attenuated the oxaliplatin-induced pain behavior. Marked increase of nNOS expression in the dorsal root ganglia (DRG) and spinal cord contributes to spinal sensory processing in CCI model [11]. More recent experiments with selective NOS inhibitors and in NOS-deficient mice revealed the nNOS to be the most important NO-producing enzyme in the spinal cord during the development and maintenance of neuropathic pain in SNL model [17]. In mice with neuropathic pain by transection of spinal nerve, an increase in nNOS activity is visualized in the superficial dorsal horn by NADPH diaphorase histochemistry [18]. Taken together, these findings suggest that NOS especially nNOS contributes to the incidence of oxaliplatin-induced mechanical allodynia.

Interestingly, our results show that both oxaliplatin-induced pain behavior and increase of NOS activity are reversed by intrathecal injection of Ro25-6981, a selective NR2B antagonist. Phosphorylation of NMDA receptor NR2B subunits increases nNOS activity in the superficial dorsal horn of mice with neuropathic pain [18]. The activation of NMDA receptor also induces glutamate release through NOS activity [19]. Thus, the NMDA receptor and NOS comprise a local circuit that amplifies the signal of pain transmission. If sustained production of these factors by repeated administration of oxaliplatin is required for maintenance of mechanical allodynia, and if their treatment-induced increase is likely to cause persistence of pain, blockade of this circuit by the NR2B antagonist would likely reduce pain excitatory neurotransmission in the spinal cord. All of these findings indicate that NR2B antagonists have analgesic effects on the oxaliplatin-induced mechanical allodynia at the spinal level.

Non-competitive NMDA receptor antagonists are used as analgesics in clinical practice, although undesirable side effects limit their utility [20]. In contrast, the restricted distribution of NR2B receptor makes them promising candidates as targets of side effect-free analgesic drugs [21]. Indeed, ifenprodil, traxoprodil (CP-101606) and Ro25-6981 are effective in inflammatory and/or neuropathic pain models in animals at doses that are not accompanied by motor effects [8,22]. In addition, ifenprodil has been used as analgesic adjuvant in clinical settings. In this study, our results showed that NMDA receptor antagonists, selective NR2B antagonists and NOS inhibitors at the effective dose had no effect on pain behavior in intact rats. Therefore, the ameliorative effects of these drugs were not attributable to non-specific sedative effects or a deficit of motor function, suggesting that the reduced pain behavior reflects a therapeutic effect on oxaliplatin-induced mechanical allodynia. Novel strategies involving NR2B antagonists may be a useful alternative or adjunct therapy for oxaliplatin-induced peripheral neuropathy.

Conclusion

Our results indicate that repeated administration of oxaliplatin induces NR2B and NOS up-regulation in the spinal cord. This up-regulation may contribute to the incidence of mechanical allodynia. Furthermore, NMDA receptor antagonists, selective NR2B antagonists and NOS inhibitors remarkably attenuated the oxaliplatin-induced pain behavior. In addition, the selective NR2B antagonist inhibited the increase of NOS activity in the spinal cord. These results suggest that activation of the NMDA-NOS pathway contributes to the incidence of mechanical allodynia induced by repeated administration of oxaliplatin.

Methods

Animals

Male Sprague-Dawley rats weighing 200-250 g (Kyudo Co., Saga, Japan) were used in the present study. Rats were housed in groups of four to five per cage, with lights on from 7:00 to 19:00 h. Animals had free access to food and water in their home cages. All experiments were approved by the Experimental Animal Care and Use Committee of Kyushu University according to the National Institutes of Health guidelines, and we followed International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues guidelines for animal research [23].

Production of neuropathy

Mechanical allodynia was induced according to the method described previously [24]. Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo, Japan). Oxaliplatin was dissolved in 5% glucose solution. The vehicle-treated rats were injected with 5% glucose solution. Oxaliplatin (4 mg/kg) or vehicle (5% glucose) was injected i.p. in volumes of 1 mL/kg twice a week for 4 weeks.

von Frey test

The mechanical allodynia was assessed by von Frey test. Each rat was placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. von Frey filaments (The Touch Test Sensory Evaluator Set; Linton Instrumentation, Norfolk, UK) ranging 1-15 g bending force were applied to the midplantar skin of each hind paw with each application held for 6 s. The paw withdrawal threshold was determined by a modified up-down method [25].

Pharmacological studies

We confirmed the incidence of mechanical allodynia on Day 24. We carried out the drug evaluation on the next day. The von Frey test was performed immediately before (0 min) and at 30, 60, 90, and 120 min after administration. (+)-MK-801 maleate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), memantine hydrochloride (Alexis Biochemicals, San Diego, CA, USA) and L-NAME (Sigma-Aldrich, Missouri, USA) were dissolved in saline and administered i.t. Ro 25-6981 (Sigma-Aldrich) and 7-nitroindazole were dissolved in 100% dimethyl sulfoxide (DMSO) and administered i.t. Ifenprodil tartrate (Wako Pure Chemical Industries, Ltd.) was suspended in 5% gum arabic solution and administered orally (p.o.). The doses of these drugs were chosen based on previous reports [8,26-28]. Behavioral test was performed blindly with respect to drug administration.

Western blotting

To investigate the functional changes in protein levels of NR2B, the L4-6 spinal cord was quickly removed on Days 5 and 25. The tissues were homogenized in a solubilization buffer containing 20 mM Tris-HCl (pH7.4, 2 mM EDTA, 0.5 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.32 M Sucrose, 2 mg/ml aprotinine, 2 mg/ml leupeptin), and the homogenates were subjected to 6% SDS-PAGE, and proteins were transferred electrophoretically to PVDF membranes. The membranes were blocked in Tris-buffered saline Tween-20 (TBST) containing 5% BSA (Sigma-Aldrich) for an additional 1 h at room temperature with agitation. The membrane was incubated overnight at 4°C with rabbit polyclonal NR2B antibody (1:5000; Upstate Biotech, NY, USA) and then incubated for 1 h with anti-rabbit IgG horseradish peroxidase (1:5000; Jackson Immuno Research Laboratories, Inc., PA, USA). The immunoreactivity was detected using Enhanced Chemiluminescence (Perkin Elmer, Massachusetts, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

To investigate the functional changes in mRNA levels of NR2B, the L4-6 spinal cord was quickly removed on Days 5 and 25. mRNA was isolated using PolyATtract® System 1000 (Promega, Corp., Wisconsin, USA). cDNA was synthesized using PrimScript® 1st strand cDNA Synthesis Kit (TaKaRa Bio, Inc., Shiga, Japan). PCR was carried out with Gene Taq (Nippon Gene, Co., Ltd., Tokyo, Japan). The oligonucleotide primers for NR2B were designed based on the sequences described by Lau et al. [29]. The sequences of PCR primers were as follows: NR2B, 5'-TCC GTC TTT CTT ATG TGG ATA TGC-3' (sense), 5'-CCT CTA GGC GGA CAG ATT AAG G-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'-YGC CTG CTT CAC CAC CTT-3' (sense), 5'-TGC MTC CTG CAC CAC CAA CT-3' (antisense) (Sigma-Aldrich). Reactions were run for 40 cycles with 95°C denaturing cycle (30 s), 63°C annealing cycle (1 min) and 72°C extension cycle (30 s) for NR2B or for 30 cycles with 94°C denaturing cycle (45 s), 53°C annealing cycle (45 s) and 72°C extension cycle (1.5 min) for G3PDH, respectively. The PCR products were subjected to electrophoresis on 2% agarose gel, and the DNA was visualized by staining with ethidium bromide under ultraviolet irradiation. Then, the intensities of PCR products were semi-quantified densitometrically by Alpha Imager 2200 (Cell Biosciences, Inc., California, USA).

NADPH diaphorase histochemistry

Animals were anaesthetized with pentobarbital (50 mg/kg) and perfused through the left cardiac ventricle with 50 mL physiological saline followed by a fixative containing 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4). The L4-6 spinal cord was removed and immersed in the fixative for 4 h and then cryoprotected overnight in 30% (w/v) sucrose in 0.1 M phosphate-buffered saline (pH 7.4). Transverse frozen sections (30 μm) were cut on a cryostat. These sections were thaw-mounted on slides and NOS activity was determined using NADPH diaphorase histochemistry as described by Mabuchi et al. [30]. The incubation was performed for 1 h at 37°C in a reaction mixture containing 0.5 mg/mL β-NADPH, 0.2 mg/mL nitroblue tetrazolium and 0.25% Triton X-100 in 0.1 M phosphate-buffered saline (pH 7.4).

Statistical analyses

Values were expressed as mean ± SEM. Results were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test to determine differences among the groups. A P value of less than 0.05 is considered as statistically significant.

Acknowledgements

Part of this study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 21590285 and 22590242).

Authors' contributions

YM, NE, TK, TY, HI and RO are responsible for experimental design. YM and HS are responsible for performance of behavioral test. YM, HS, TK and SU are responsible for performance of Western blot, PCR and NADPH diaphorase staining. YM, NE, HS, TK and RO are responsible for writing the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 1 November 2010 Accepted: 20 January 2011
Published: 20 January 2011

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doi:10.1186/1744-8069-7-8
Cite this article as: Mihara *et al.*: Involvement of spinal NR2B-containing NMDA receptors in oxaliplatin-induced mechanical allodynia in rats. *Molecular Pain* 2011 **7**:8.

Surg Today (2010) 40:1105–1111
DOI 10.1007/s00595-010-4388-8

SURGERY TODAY

Review Article

The Effect of Traditional Japanese Medicine (Kampo) on Gastrointestinal Function

ERITO MOCHIKI, MITSUHIRO YANAI, TETSURO OHNO, and HIROYUKI KUWANO

Department of General Surgical Science, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan

Abstract
Traditional Japanese medicine (Kampo) is used to treat various disorders of the gastrointestinal tract in Japan, where it is fully integrated into the modern healthcare system. Recently, scientific research on herbal medicine in Japan has been reported in English journals. The objective of the current review is to introduce two traditional Japanese medicines and to provide evidenced-based information regarding their use. Daikenchuto, which consists of three different herbs, is the most frequently prescribed traditional Japanese medicine in Japan. Daikenchuto stimulates gastrointestinal motility though a neural reflex involving presynaptic cholinergic and 5-HT3 receptors. Daikenchuto improves postoperative bowel motility and postoperative ileus. Furthermore, it is reported to cause an increase in gastrointestinal hormones (motilin, vasoactive intestinal peptide, and calcitonin gene-related peptide) and intestinal blood flow. Rikkunshito, a traditional Japanese medicine consisting of eight herbs, is thought to stimulate gastrointestinal motility and ghrelin secretion. Rikkunshito is effective for improving the symptoms of functional dyspepsia, gastroesophageal reflux disease, and cisplatin-induced anorexia and vomiting. Traditional Japanese medicine has the potential to be used successfully in the treatment of gastrointestinal disorders. Details regarding the physiological and clinical effects of traditional Japanese medicine must be further examined in order to become more widely accepted in other countries.

Key words Traditional Japanese medicine · Kampo · Rikkunshito · Daikenchuto · Gastrointestinal function

Reprint requests to: E. Mochiki
Received: June 29, 2010 / Accepted: July 26, 2010
Recent data for this review were collected by Medline searches using the following keywords: "traditional Japanese medicine," "Japanese herbal medicine," "herbal medicine," and "kampo."

Introduction

Traditional Japanese medicine, which includes Kampo, acupuncture, and acupressure, has been used for 1500 years. The use of Kampo, or herbal medicine, is based on extensive experience with herbal combinations accumulated in East Asia since ancient times. Kampo is intended to boost the body's own healing power and help restore its natural balance. Traditional Japanese medicine is widely practiced in Japan, where it is fully integrated into the modern health-care system.

The National Center for Complementary and Alternative Medicine, established at the National Institutes of Health in the United States in October 1998, recategorized traditional medicine as "complementary and alternative medicine" following the increased interest in non-Western medicine.[1] Recently, scientific research on herbal medicine in Japan has been reported in English-language journals, and several rigorous clinical and basic research studies have confirmed the effects of traditional Japanese medicine. As a result, the United States Food and Drug Administration began to pay more attention to traditional medicine, especially traditional Japanese medicine, noting its exceptionally high quality and standardized ingredients. The objective of this review is to introduce two traditional Japanese medicines and to provide evidenced-based information regarding their effects and use.

Gastrointestinal Function (Motility)

The gastrointestinal tract has distinct contractile patterns. Gastrointestinal motility is clearly divided into two phases: the interdigestive state and the postprandial state. During the interdigestive state, the gastrointestinal tract exhibits a characteristic motor pattern called interdigestive migration motor contraction (IMC),[2] which consists of four phases, with a combined duration