Table 4. Nonhematological toxicity ($n = 60$)

Adverse effect	Toxicity grade					
	G0	G1	G2	G3	G4	G3+G4
Fever	48 (80.0)	8 (13.3)	3 (5.0)	1 (1.7)	0 (0.0)	1 (1.7)
Febrile neutropenia	58 (96.7)	0 (0.0)	0 (0.0)	2 (3.3)	0 (0.0)	2 (3.3)
Infection	57 (95.0)	0 (0.0)	0 (0.0)	3 (5.0)	0 (0.0)	3 (5.0)
Fatigue	31 (51.7)	14 (23.3)	12 (20.0)	3 (5.0)	0 (0.0)	3 (5.0)
Diarrhea	51 (85.0)	5 (8.3)	2 (3.3)	2 (3.3)	0 (0.0)	2 (3.3)
Nausea	32 (53.3)	16 (26.7)	9 (15.0)	3 (5.0)	0 (0.0)	3 (5.0)
Vomiting	49 (81.7)	4 (6.7)	7 (11.7)	0 (0.0)	0 (0.0)	0 (0.0)
Anorexia	26 (43.3)	16 (26.7)	12 (20.0)	6 (10.0)	0 (0.0)	6 (10.0)
Stomatitis	44 (73.3)	11 (18.3)	5 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)
Peripheral neurotoxicity	17 (28.3)	17 (28.3)	22 (36.7)	3 (5.0)	1 (1.7)	4 (6.7)
Allergy	54 (90.0)	4 (6.7)	0 (0.0)	0 (0.0)	2 (3.3)	2 (3.3)
Alopecia	47 (78.3)	13 (21.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Rash	58 (96.7)	2 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hand–foot syndrome	55 (91.7)	4 (6.7)	1 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)
Hyperpigmentation	59 (98.3)	1 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Data are number of patients (%)

Table 5. Hematological toxicity

Adverse effect	Toxicity Grade					
	G0	G1	G2	G3	G4	G3+G4
Leukopenia	15 (25.0)	13 (21.7)	22 (36.7)	10 (16.7)	0 (0.0)	10 (16.7)
Neutropenia	10 (17.2)	6 (10.3)	14 (24.1)	20 (34.5)	8 (13.8)	28 (48.3)
Thrombocytopenia	17 (28.3)	34 (56.7)	8 (13.3)	1 (1.7)	0 (0.0)	1 (1.7)
Anemia (Hb)	6 (10.0)	43 (71.7)	9 (15.0)	2 (3.3)	0 (0.0)	2 (3.3)
Total bilirubin	44 (74.6)	12 (20.3)	3 (5.1)	0 (0.0)	0 (0.0)	0 (0.0)
ALT	34 (56.7)	19 (31.7)	6 (10.0)	1 (1.7)	0 (0.0)	1 (1.7)
AST	19 (31.7)	33 (55.0)	7 (11.7)	1 (1.7)	0 (0.0)	1 (1.7)
ALP	26 (45.6)	29 (50.9)	2 (3.5)	0 (0.0)	0 (0.0)	0 (0.0)
Creatinine	53 (89.8)	5 (8.5)	1 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)

Data are number of patients (%)

Discussion

Since its approval for use in Japan in April 2005, the attitude toward L-OHP has largely been based on evidence produced by large phase III studies carried out in Western countries. The results of the present prospective study demonstrate the efficacy and feasibility of FOLFOX4 as first-line treatment for Japanese patients with advanced or metastatic colorectal cancer, similar to what has already been demonstrated in Western populations. The response rate and median PFS in patients receiving first-line treatment were 45%–53.7% and 8.7–9.4 months, respectively.[7,8,12,13] Although both the RR (34.5%, 95% CI: 22.5%–48.1%) and median PFS (7.0 months, 95% CI: 5.1–9.8 months) in our study were slightly lower than those observed in previous clinical studies, with a CI of 95%, the difference was not significant. The tumor control rate in our study, at 82.8%, was comparable to the rate of 80.2%–90.7% seen in previous clinical studies.[7,8,12,13]

The most impressive finding from this study was an OS in excess of 30 months. This was probably due to the fact that most of the patients received further chemotherapy including irinotecan or biological agents after they failed to respond to FOLFOX4. The overall survival in patients with advanced colorectal cancer was reported to be strongly correlated with the percentage of patients who received the three drugs fluorouracil, irinotecan, and L-OHP in the treatment of their disease.[14,15] We also investigated the relationship between the therapeutic regimen used after FOLFOX4 and the survival time in 52 patients for whom information was available. In 32 patients, treatment included molecularly targeted agents, while in 20 patients it did not. The median survival times were 40.1 and 18.8 months in groups treated with and without the molecularly targeted agents, respectively (data not shown). The results of this study indicate that the FOLFOX4 regimen is beneficial as first-line therapy for advanced or metastatic colorectal cancer in the Japanese population, and

that subsequent administration of molecularly targeted agents provides further benefits.

In this study, although grade 3/4 neutropenia occurred in 48.3% of the patients assigned to receive L-OHP, it was nonfebrile. Moreover, no patient was affected by grade 3/4 vomiting or mucositis, and diarrhea affected only 3.3% of the patients. The cumulative dose-limiting toxicity of L-OHP is peripheral sensory neuropathy, which reportedly occurs in about 70% of patients, usually resolves a few months after discontinuation of treatment, and may be exacerbated by cold stimulation.[5,7] Severe paresthesia occurs in 16% of patients, affecting function.[7] In our study, 3 patients declined further FOLFOX4 treatment because of unacceptable paresthesia, even though it was graded moderate. In general, the paresthesia was reversible upon dose reduction or cessation of L-OHP. Overall, half of the patients experienced mild to moderate paresthesia at a median of 9 cycles.

A small number of case reports describing anaphylactic reactions to L-OHP have been published.[16–18] Brandi et al.[19] reported their experience with L-OHP hypersensitivity in a Caucasian population, where 13% of 124 patients experienced hypersensitivity reactions. The incidence of hypersensitivity reactions to L-OHP in our study (3.4%) was lower than that reported in Western countries. However, the median number of doses received prior to the hypersensitivity reactions in the Brandi et al. study was 9 (range 2–17) compared with 8 (range 6–10) in ours. The mechanism of hypersensitivity reactions to L-OHP has yet to be determined, but could be similar to that of the immunoglobulin E-mediated reactions in patients with cisplatin or carboplatin allergies.

In conclusion, the FOLFOX4 regimen demonstrated good efficacy in a Japanese population, with an acceptable overall toxicity profile. This suggests that it should be used as a standard first-line therapy for patients with advanced or metastatic colorectal cancer in Japan. Further studies will be necessary to investigate its potential benefits when used in combination with targeted agents such as bevacizumab, cetuximab, and panitumumab.

Contributors

Y.M. was the principal investigator. H.B., S.T., and Y.K. were responsible for the study conception and design. N.H., T.O., M.Y., H.H, F.K., H.H., K.N., F.F., Y.O., S.K., M.I., and HS provided patients. Y.E., Y.K., A.E., E.O., K.S., and H.B. performed the data review. Y.E. collected and collated the data. S.T. designed the study based on statistical parameters, supervised the data management, and statistically analyzed the data. H.B., N.H., S.T., Y.K., and Y.M. interpreted the data. The manuscript was approved by all authors.

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特集1　胃癌化学療法

進行胃癌におけるS-1＋Docetaxelによる集学的治療戦略*

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Key Words : S-1, docetaxel, gastric cancer, adjuvant chemotherapy

はじめに

胃癌に対する手術療法は最も根治性が望める治療法である．胃癌の定型手術は，胃癌治療ガイドライン第3版(2010年)[1]に，胃の2/3以上切除とD2リンパ節郭清〔D1：No. 1～7＋No. 8a, 9, 11p, 12a(胃全摘術ではさらに＋No. 10, 11d)〕を施行する手術と記載されている．適応の原則は，他臓器浸潤がなく，D2で癌の遺残がないと期待できる症例であり，その具体的適応条件について治癒切除可能なT2(MP)以深の腫瘍，およびcN＋のT1(M, SM)腫瘍とされている．D2を超える拡大リンパ節郭清は非定型手術に分類され，予防的な大動脈周囲リンパ節(No. 16)の意義はわが国のRCT(JCOG-9501)[2]で否定された．

一方，進行・再発胃癌に対する化学療法は，JCOG-9912試験[3]，SPIRITS試験[4]の結果からS-1＋cisplatin(CDDP)療法が現時点で推奨できる[1]．S-1＋CPT-11の優越性は証明されず[5]，S-1＋docetaxelは臨床第III相試験(JACCRO GC-03試験)[6]の結果を待っているところである．

われわれは，高い奏効率を示しkey drugとなるS-1の併用療法の組み合わせとしてdocetaxel(DOC)に注目し，併用療法の臨床第I, II相試験を進行・再発胃癌に対して行い，さらに術前・術後補助化学療法の臨床第II相試験を進めてきた[7]．

基礎的検討

*In vitro*においてDOCは5-FU耐性細胞に対しても交叉耐性をもたないことが報告されている[8]．単剤での臨床第II相試験では奏効率23.4％であり，前化学療法が行われた症例に対しても21.7～21.9％と同程度の効果を上げている[9][10]．

Nude ratの系でヒト胃癌株SC-2, St-40, SC-4を移植し，control群，S-1単独群，DOC単独群，S-1＋DOC併用群を比較した[11]．S-1とDOCの併用のタイミングをみるために，S-1はday 1～14とするなかでDOC投与はday 1とday 8の二通りのスケジュールを検討した．day 1にDOCを先行投与する方がday 8に投与するよりも相乗的な抗腫瘍効果が強く，体重減少からみた副作用が軽度であった．相乗効果の機序として，Wadaら[12]は，胃癌細胞株TKM-1においてDOC処理にてTS, DPDの蛋白発現が経時的に減少し，この傾向は5-FUおよびDOC同時処理にて増強すると報告している．

* Multimodality therapy using S-1 and docetaxel combined chemotherapy for advanced gastric cancer.

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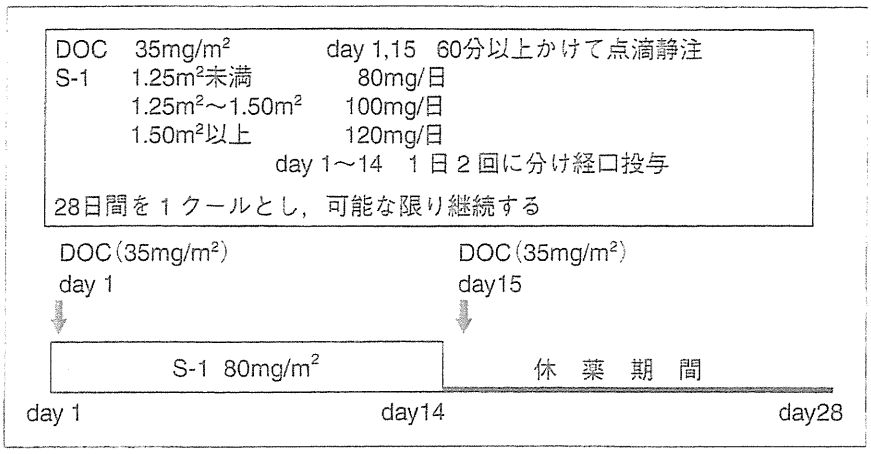

図1　S-1＋docetaxcel 2週ごとスケジュールPII試験
われわれのプロトコールはdocetaxelを35mg/m²でday 1, day15に投与する．S-1は2週間投与，2週休薬となる．1サイクルごとのdocetaxelの量が70mg/m²となり，抗腫瘍効果が期待される．

臨床第Ⅰ相試験

S-1＋biweekly DOCの治療スケジュールにて両者の併用を検討した[13]．保険認可でのDOC投与(60mg/m²，3～4週ごとの点滴静注)でのgrade 3以上の白血球減少や好中球減少などの重篤な副作用を，DOCを分割投与することで軽減することを意図した．S-1の用量は体表面積に応じて80～120mg/m² 2×(day 1～14)と単剤での保険認可用量に固定し，DOCはday 1およびday15に静脈注射を行うこととした．DOCはレベル1の40mg/m²から開始したが，3例のdose limiting toxicity(grade 4の白血球減少1例，grade 3の好中球減少によるday 15投与延長2例)を経験した．レベル－1の30mg/m²，レベル0の35mg/m²での検討を行い，推奨用量は35mg/m²に決定した．

臨床第II相試験

臨床第Ⅰ相試験の結果をもとにDOCはday 1, 15に35mg/m²投与し，S-1は80mg/m²を14日間投与14日間休薬とし，28日間を1コースとした(図1)臨床第II相試験を行った[14]．35例が登録され，grade 3以上の主な有害事象は好中球減少(22.9%)，白血球減少(11.4%)，食欲不振(11.4%)などであり，いずれも外来通院を中心として対応可能であった(表1)．complete response(CR) 1例，partial response(PR) 13例，stable disease(SD) 13例，progressive disease(PD) 3例，not evaluable(NE) 5例でPR以上の奏効率40%，SD以上の病勢コントロール率77%であった．標的病変別の奏効率を

表1　有害事象(n=35)

有害事象	NCI-CTC v2.0 Grade 1	2	3	4	G 3/4
全体*	1	17	11	4	42.9%
血液学的毒性					
血色素量減少	3	8	3		8.6%
白血球数減少	5	16	3	1	11.4%
好中球数減少	4	13	4	4	22.9%
血小板数減少	1	1	1		2.9%
非血液学的毒性					
AST	1				
ALT	1				
食欲不振	7	6	4		11.4%
悪心	8	2		–	
嘔吐	3	2			
下痢	6	1			
口内炎	5	3	3		8.6%
味覚障害	4				
鼻出血	1				
腹痛	1				
発熱	2				
色素沈着	6	2	–	–	
発疹/落屑	1	2			
掻痒感	1				
脱毛	10	2	–	–	
全身倦怠感	9	2	1		2.9%
流涙	1				
結膜炎		2			
咽頭炎		2			
頭痛	1				

* Grade 0の症例2例．

表２　標的病変別の臨床効果(n=35)

標的病変	CR	PR	SD	PD	NE	奏効率(%)	Disease control rate(%)
肝	0	6	7	1	3	42.9(6/17)	76.5
肺	0	1	1	0	0	50.0(1/2)	100
腹部リンパ節	1	5	3	2	2	46.1(5/12)	69.2
肝＋腹部リンパ節	0	1	2	0	0	33.3(1/3)	100
計	1	13	13	3	5	40.0(14/35)	77.1

表３　組織型別の臨床効果(n=35)

組織型	CR	PR	SD	PD	NE	奏効率(%)	Disease control rate(%)
高分化型	0	3	3	0	1	42.9(3/7)	85.7
中分化型	1	4	6	2	2	33.3(5/15)	73.3
低分化型：充実型	0	3	3	0	1	42.9(3/7)	85.7
低分化型：非充実型	0	3	1	1	0	60.0(3/5)	80
結液癌	0	0	0	0	1	−	−
計	1	13	13	3	5	40.0(14/35)	77.1

みてみると，肝42.9%，肺50.0%に加えて腹部リンパ節も46.1%と良好な成績を示している(表2)．S-1＋docetaxelは転移先の臓器の違いにかかわらず効いているように思われる．組織型では，分化型にも低分化型にも高い奏効率を示しており(表3)，難治性の腹膜播種にも効果が期待される．無増悪生存期間は4.5か月，生存期間中央値は14.2か月であった(図2)．1年生存率が51.4%，2年生存率が24.6%であった．

国内でのS-1＋DOC併用療法の臨床第II相試験の比較を表4に示す[14)~16)]．biweeklyにDOCを投与する方法は17.5mg/m²/wkと高いdose intensityを目指す治療法である．実際に投与した量のdose intensityは14.6mg/m²/wkであった．有害事象によりday15のDOC投与ができない場合は，速やかに減量した方が休薬期間を延ばすよりも抗腫瘍効果が得られると考えられた．重篤な有害事象は明らかに少なく，外来を中心としたmanagementが可能である．生存期間中央値は14.2か月であり，triweeklyの14.3か月とほぼ同等である．本試験では35例中14例(40%)がCPT-11を中心とした2 nd lineの化学療法を施行できた．重篤な有害事象が少ないことと，病勢コントロール率が高いことが2 nd lineへの移行を可能にし，全生存期間の延長につながったものと考えられる．

化学療法が奏効した3例については，手術を施行できた．cT3N3M1 cStage IV, cT4N2M0 cStage IV, cT3N3M0 cStage IVの3例であり，

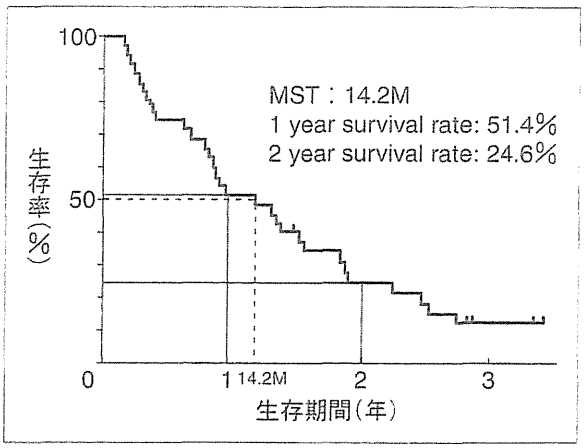

図２　全生存期間(overall survival)
全生存期間の中央値(MST)は14.2か月と他のスタディとほぼ同様の結果だった．1年生存率が51%，2年生存率が24%だった．

術後の化学療法も行い，2例は1,000日以上の生存が得られている．

S-1＋DOC併用療法は組織型や転移臓器に関係なく抗腫瘍効果が得られ，病勢コントロールが可能で生存期間の延長が望める．さらなる治療効果の改善を目指して，手術を含む集学的治療を検討した．

術後補助化学療法

日本における標準術式であるD2リンパ節郭清を伴った胃切除術を施行したステージIIまたはIIIの胃癌患者を，術後にS-1による補助療法を行う群と，手術単独群に無作為に割り付けたACTS-GC

表4　S-1＋docetaxel臨床第II相試験の比較

項目	biweekly[13]	triweekly[14]	monthly[15]
症例数	35	48	46
docetaxelの予定dose intensity	17.5mg/m²/wk[a]	13.4mg/m²/wk[b]	10.0mg/m²/wk[c]
サイクル数中央値(range)	3(1-19)[d]	4(1-17)[e]	3(1-6)[d]
Grade 3/4 好中球減少—%	23	58	67
発熱性好中球減少症—%	0	8	0
奏効率—%	40	56	46
全生存期間—月	14.2	14.3	14.0

a：実投与量のdose intensityは14.6mg/m²/wk．b：次コースの開始が272サイクル中，56サイクルで遅れ，遅延の中央値は7日(1～14日)であった．c：docetaxelのdose intensityの比率の中央値は99%(range, 75～101)．d：1サイクル＝4週間．e：1サイクル＝3週間．

試験中の中間解析結果で，3年全生存率はS-1群で80.1%，手術単独群で70.1%であった．効果・安全性評価委員会の勧告に基づいて試験は中止され，T3(SS)N0を除くStage II/III胃癌の術後補助化学療法としてS-1の術後1年間投与が標準治療となった．Stage IIIの再発率は高く，S-1＋α療法を術後または術前に行う意義を検討することが望まれる(図3)．

根治切除を行ったStage II, IIIA, IIIB胃癌の術後にDOCをday 1に40mg/m²投与し，S-1は80mg/m²を14日間投与7日間休薬とし，21日間を1コースとするtriweeklyの投与法を採用した(図4)．術後にS-1＋docetaxel療法を4コース行い，その後S-1投与を1年間続ける．この第II相試験はprimary endpointを治療完遂率とし，目標20例に対して22例の登録が成された．有害事象は，好中球減少，下痢，食欲不振，悪心等のgrade 3が27%(6/22)，好中球減少のgrade 4が5%(1/22)であり，外来で十分対応可能であった．主要評価項目である4コースの治療完遂率は68%(15/22)であった．7人の治療中止理由は，grade 3の食欲不振(2人)，grade 3の下痢および急性腸炎(1人)，grade 4の好中球減少(1人)，被験者からの試験継続拒否(2人)，医師による中止選択(1人)であった．

胃癌術後の補助化学療法としてS-1＋DOC併用療法は外来で対応可能であり，安全性も確認でき，重篤な有害事象は少なかった．

術前補助化学療法

胃癌治療ガイドライン第3版(2010年)[1]で，術前補助化学療法は「臨床研究としての治療法」と

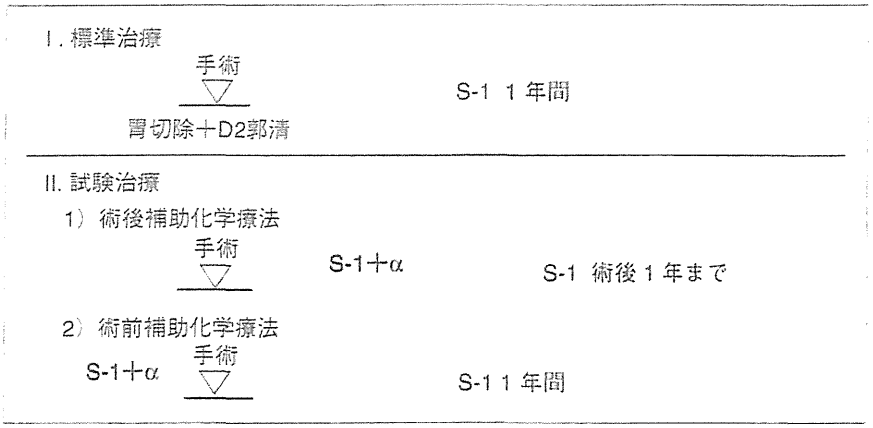

図3　Stage III胃癌に対する治療戦略
切除可能胃癌に対する新しい治療戦略としては，①S-1単剤よりも効果の高い術後補助化学療法を開発する②術前化学療法を開発するという2つの戦略がある．どちらの戦略を考えるとしても，そのベースとなるのは現在の標準治療である，胃切除＋D2郭清＋術後S-1投与であり，術前化学療法を考える際は，この標準治療に術前化学療法を追加する意義があるか否かを議論する必要がある．

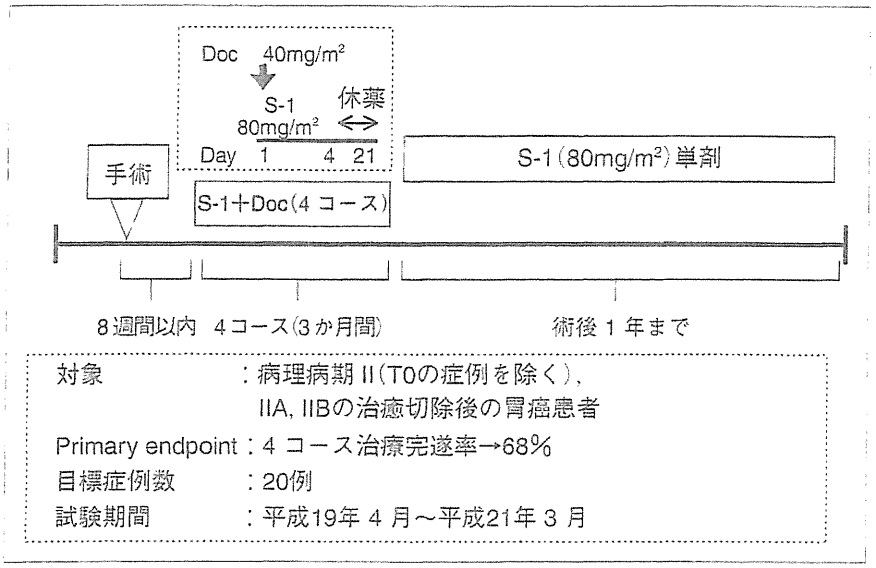

図4 術後補助化学療法としてのS-1＋Doc併用療法
術後補助化学療法としてのS-1＋DOC併用療法は施行可能であり，有用性が期待できる．

位置づけられている．術前補助化学療法は術後補助化学療法と比べて，以下の利点がある．①原発巣による化学療法の効果判定が可能である．②術前のため，術後に比べてコンプライアンスが高い．③化学療法によってリンパ節転移などが縮小し，down stagingが期待できる．④微小転移に対して早期から化学療法を行えるため，根治を目指せる可能性がある．

根治切除可能なStage IIIA, IIIB, IV(T4N2のみ)胃癌を対象とした．診断的腹腔鏡検査を行って腹膜播種がないことを確認した後にDOCをday 1, 15に35mg/m²投与し，S-1は80mg/m²を14日間投与14日間休薬とし，28日間1コースで2コース行う臨床第II相試験を開始した(図5)．primary endpointは病理学的奏効割合とした．目標症例数45例で47例の症例登録が成された．術前化学療法を中止基準に抵触せずに2コース完遂し(減量，休薬は可)，原病の増悪がなく，プロトコール手術を完了できたプロトコール治療完遂率は79%(37/47)で，術前化学療法のいかんにかかわらず，プロトコール手術を完了できた根治切除可能例は94%(44/47)であった．主要評価項目のgrade 1b以上の病理学的奏功割合は47%であり，grade 3の著効例も4%(2/47)に認められた．術前化学

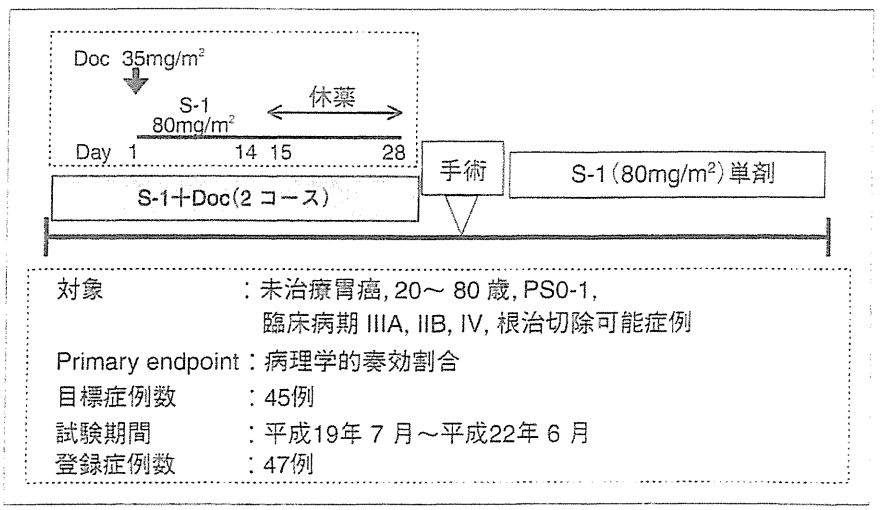

図5 術前補助化学療法としてのS-1＋Doc併用療法

療法として重篤な有害事象も認められず，術後合併症も腹腔内膿瘍(12.8%)や膵液漏(10.6%)を認めたが許容範囲であり，手術への悪影響も少なかった．S-1＋DOC術前補助化学療法は，進行胃癌の治療戦略において有望な治療法であることが示唆された．

結　語

進行胃癌に対するS-1＋DOC併用療法は病勢コントロール率が高く，副作用が少なく，有意な生存期間の延長をもたらした．術後補助化学療法や術前補助化学療法など，手術療法と効果的に組み合わせることで進行胃癌のさらなる治療成績向上が期待できると考えられた．

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Prevention of oxaliplatin-induced mechanical allodynia and neurodegeneration by neurotropin in the rat model

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ABSTRACT

Oxaliplatin is a key drug for colorectal cancer, but it causes acute peripheral neuropathy (triggered by cold) and chronic neuropathy (sensory and motor neuropathy) in patients. Neurotropin, a non-protein extract from the inflamed rabbit skin inoculated with vaccinia virus, has been used to treat various chronic pains. In the present study, we investigated the effect of neurotropin on the oxaliplatin-induced neuropathy in rats.

Repeated administration of oxaliplatin caused cold hyperalgesia from Day 5 to Day 29 and mechanical allodynia from Day 15 to Day 47. Repeated administration of neurotropin relieved the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia, and inhibited the oxaliplatin-induced axonal degeneration in rat sciatic nerve. Neurotropin also inhibited the oxaliplatin-induced neurite degeneration in cultured pheochromocytoma 12 (PC12) and rat dorsal root ganglion (DRG) cells. On the other hand, neurotropin did not affect the oxaliplatin-induced cell injury in rat DRG cells. These results suggest that repeated administration of neurotropin relieves the oxaliplatin-induced mechanical allodynia by inhibiting the axonal degeneration and it is useful for the treatment of oxaliplatin-induced neuropathy clinically.

1. Introduction

Oxaliplatin, a platinum-based chemotherapeutic agent, is widely used for colorectal cancer. However, it causes severe acute and chronic peripheral neuropathies. Acute neuropathy is peculiar to oxaliplatin and includes acral paresthesias enhanced by exposure to cold (Cassidy and Misset, 2002; Extra et al., 1998; Pasetto et al., 2006; Quasthoff and Hartung, 2002). It has been thought that the acute neuropathy is not due to morphological damage of the nerve (de Gramont et al., 2002; Wilson et al., 2002) and is due to alternations of voltage-gated Ca^{2+} and K^+ channels (Adelsberger et al., 2000; Benoit et al., 2006; Grolleau et al., 2001; Kagiava et al., 2008). On the other hand, the chronic neuropathy is characterized by loss of sensory and motor neuropathy after long-term treatment of oxaliplatin and it is similar to cisplatin-induced neurological symptom (Pasetto et al., 2006). Oxaliplatin causes the damage of the cell bodies (Cavaletti et al., 2001; Donzelli et al., 2004; McKeage et al., 2001; Scuteri et al., 2009; Ta et al., 2006), inhibition of neurite outgrowth (Luo et al., 1999), alterations in nuclearus morphology (Cavaletti et al., 2001), and selective atrophy of subpopulation of dorsal root ganglion (DRG) neurons (Jamieson et al., 2005). Oxaliplatin is metabolized to oxalate and dichloro(1,2-diaminocyclohexane) platinum [Pt(dach)Cl_2] (Graham et al., 2000). Recently, we demonstrated the involvement of oxalate in oxaliplatin-induced cold hyperalgesia but not mechanical allodynia, and preventive effect of pre-administration of Ca^{2+} or Mg^{2+} on the cold hyperalgesia in rats (Sakurai et al., 2009). On the other hand, pre-administration of Ca^{2+} or Mg^{2+} could not attenuate the mechanical allodynia which is related to Pt(dach)Cl_2 (Sakurai et al., 2009).

Neurotropin is a non-protein extract derived from the inflamed skin of rabbits inoculated with vaccinia virus. Neurotropin is clinically used to treat various chronic pain conditions, including post herpetic neuralgia and hyperesthesia of subacute myelo-optic neuropathy (SMON). Moreover, National Institute of Nursing Research (NINR) in the United States is now examining the safety and effectiveness of neurotropin for preventing or easing pain associated with fibromyalgia and treating chronic pain after injury to a limb or a large nerve. We previously reported that repeated administration of neurotropin reverses the paclitaxel-induced neuropathy without affecting anticancer activity in rats (Kawashiri et al.,

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2009). However, the effect of neurotropin on the oxaliplatin-induced neuropathy remains unexplored. Accordingly, we examined the effect of neurotropin on the oxaliplatin-induced cold hyperalgesia and mechanical allodynia in rats.

2. Methods

2.1. 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 800 to 2000 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 (Zimmermann, 1983).

2.2. Drug administration

Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo, Japan). Sodium oxalate was provided by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Neurotropin was a generous gift from Nippon Zoki Pharmaceutical Co. (Osaka, Japan). Oxaliplatin and sodium oxalate were dissolved in 5% glucose solution. The vehicle-treated rats were injected with 5% glucose solution. Oxaliplatin (4 mg/kg), sodium oxalate (1.3 mg/kg) or vehicle was injected i.p. in volumes of 1 mL/kg twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22 and 23). Neurotropin [200 Neurotropin Unit (NU)/kg] was administered p.o. in volume of 10 mL/kg three times a week for 4 weeks (Days 1, 2, 3, 8, 9, 10, 15, 16, 17, 22, 23 and 24). Behavioral tests were performed blind with respect to drug administration. The doses of oxaliplatin, sodium oxalate and neurotropin followed previous reports (Kawashiri et al., 2009; Ling et al., 2007; Sakurai et al., 2009).

2.3. von Frey test for mechanical allodynia

The mechanical allodynia was assessed by von Frey test. The von Frey test was performed before the first drug administration (on Day 1) and on Days 5, 8, 12, 15, 19, 22, 26, 29, 33, 40, 47 and 54. On Days 5, 15 and 22, test was performed before drug administration. 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. 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. Fifty percent paw withdrawal thresholds were determined by a modification of up-down method that described by Chaplan et al. (1994). First, each hind paw was touched with some filaments from 1 g up to the force that rat exhibited the withdrawal response, in ascending order. Next, the paw was touched with some filaments from 15 g down to the force that rat did not exhibit the response, in descending order. These up and down steps were repeated three times. Fifty percent thresholds were determined by average of the weakest force in each up or down step.

2.4. Acetone test for cold hyperalgesia

The cold hyperalgesia was assessed by acetone test. The acetone test was performed before the first drug administration (on Day 1) and on Days 5, 8, 12, 15, 19, 22, 26, 29, 33, 40, 47 and 54 according to the method described by Flatters and Bennett (2004). On Days 5, 15 and 22, test was performed before drug administration. 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. Fifty microlitre of acetone (Wako Pure Chemical Ltd., Osaka, Japan) was sprayed onto the plantar skin of each hind paw three times with a Micro Sprayer® (Penn Century Inc., PA, USA), and rats were observed for 40 s from the start of the acetone spray. The number of elevation of each hind paw was recorded.

2.5. Assay of sciatic nerve axonal degeneration

On Day 5 or 25, sciatic nerves were harvested from rats anesthetized with sodium pentobarbital. Nerves were fixed in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 4 °C) for 4 h followed by wash with 0.1 M phosphate buffer. After 8% (w/v) sucrose-substitution, samples were embedded in Epon. Each section was stained with toluidine blue. Sample sections were evaluated using light microscopy (BX51; Olympus Corp., Tokyo, Japan).

2.6. Assay of cell injury in DRG neurons

The cell injury, especially nuclear damage, was stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL). On Day 5 or 25, L5 DRG neurons were harvested from rats anesthetized with sodium pentobarbital. DRG neurons were fixed in 4% (w/v) paraformaldehyde for 30 min. Samples embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) were frozen and sliced. Each section was permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate on ice. TUNEL stain was carried out using a commercial assay kit (Cell Death Detection kit, Roche Applied Science, Tokyo, Japan), according to the manufacturer's instructions. Sample sections were evaluated using fluorescence microscopy (BX51; Olympus Corp., Tokyo, Japan).

2.7. Cell lines and cultures

PC12 was obtained from the American Type Culture Collection (Walkersville, MD, USA). L 4-5 DRG cells were removed from male Sprague–Dawley rats (6 weeks old), which anesthetize with sodium pentobarbital, and primary cultured. Ganglia was incubated with 0.125% (w/v) collagenase type 1 (Worthington Biochemical Corp., NJ, USA) at 37 °C for 90 min followed by incubation with 0.25% (w/v) trypsin–EDTA (Gibco BRL, USA) for 30 min. PC12 cells were grown in RPMI 1640 medium (MP Biomedicals Inc., CA, USA) supplemented with 2 mM L-glutamine, 10% horse serum, and 5% fetal bovine serum (FBS). DRG cells were grown in Dulbecco's modified Eagle's medium (MP Biomedicals Inc.) with 2 mM L-glutamine and 10% FBS. Both cell lines were cultured on 80 cm^2 tissue culture flasks (Nunc Apogent Co., Roskilde, Denmark) at 37 °C in an air supplemented with 5% CO_2 under humidified conditions.

2.8. Assay of cultured PC12 and DRG neurite outgrowths

PC12 is the rat pheochromocytoma and characterized by neuronal differentiation by nerve growth factor (NGF). PC12 cells have been used as the model of neurodegeneration such as paclitaxel- or cisplatin-induced neurite degeneration (Pisano et al., 2003; Kawashiri et al., 2009). PC12 cells were seeded at a density of 1×10^4 cells/cm^2 onto 24 well plates (Falcon, Becton Dickinson Co., Ltd., NJ, USA) and were used for experiments on the following day. Neurite outgrowth in PC12 cells was induced by 10 μM forskolin (Carbiochem, EMD Chemicals Inc., Darmstadt, Germany) at 21 h before drug exposures. DRG cells were seeded onto 24 well plates and were cultured for a week so that neurites were extended. Both cell types were exposed to oxaliplatin (0.3, 1, 3, 10

or 30 μM), sodium oxalate (0.3, 1, 3, 10 or 30 μM) and neurotropin (0.001, 0.003, 0.01 or 0.03 NU/mL) for 168 h. After incubation with drugs, dead cells were stained with trypan blue (Gibco BRL, NY, USA). Cells were monitored by a phase contrast microscope and neurite lengths in living cells were measured by analysis software (Image J 1.36; Wayne Rasband, National Institutes of Health, MD, USA).

2.9. Assay of cell viability in cultured DRG cells

DRG cells were seeded onto 24 well plates and were cultured for a week so that neurites were extended. Cells were exposed to oxaliplatin (0.3, 1, 3, 10 or 30 μM), sodium oxalate (0.3, 1, 3, 10 or 30 μM) and neurotropin (0.001, 0.003, 0.01 or 0.03 NU/mL) for 168 h. The cell viability was assessed by the mitochondrial activity in reducing WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to formazan. At 168 h after incubation with oxaliplatin and neurotropin, the cells were washed with phosphate-buffered saline, then 210 μL of serum-free medium and 10 μL of WST-8 assay solution (Cell Counting Kit-8; Dojindo Laboratory, Kumamoto, Japan) were added and incubated for 1 h at 37 °C in humidified air supplemented with 5% CO_2. The incubation medium was carefully taken and transferred to 96 well flat-bottom plastic plates (Corning Costar, Corning, NY, USA). The amount of formed formazan dye was measured from the absorbance at 450 nm with a reference wavelength of 620 nm using a microplate reader (Immuno-mini NJ-2300; Inter Medical, Tokyo, Japan).

2.10. Statistical analyses

Values were expressed as the mean ± SEM. The values were analysed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post hoc test to determine differences among the groups. A probability level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Pain behavior

No deterioration in general status was observed, and no rats in all groups died during the course of the experiment.

Before the first oxaliplatin injection, there were no significant differences in withdrawal thresholds in all groups in the von Frey test (Fig. 1A). Oxaliplatin (4 mg/kg, i.p.) significantly reduced the withdrawal threshold compared with vehicle on Days 15, 19, 22, 26, 29, 33, 40 and 47 ($P < 0.01$). However, no significant difference in withdrawal threshold compared to the vehicle group was observed on Day 54. Repeated administration of neurotropin (200 NU/kg) significantly inhibited the oxaliplatin-induced reduction of the withdrawal threshold on Days 15, 19, 22, 26, 29, 33 and 40 ($P < 0.01$).

In the acetone test, there were no significant differences in number of withdrawal responses in all groups before the first oxaliplatin injection (Fig. 1B). Oxaliplatin significantly increased the number of withdrawal responses compared with vehicle on Days 5, 8, 12, 15, 19, 22, 26 and 29 ($P < 0.01$). However, no significant difference in withdrawal responses compared to the vehicle group was observed on Day 33. Repeated administration of neurotropin significantly inhibited the oxaliplatin-induced increase of the number of withdrawal responses on Day 5 ($P < 0.05$). However, neurotropin did not affect the oxaliplatin-induced increase of the number of withdrawal responses on Days 8, 12, 15, 19, 22, 26 and 29.

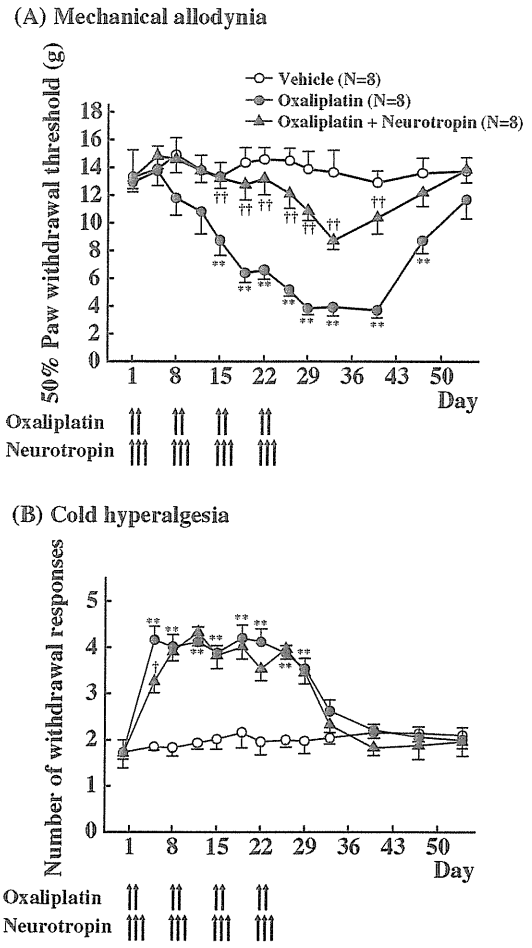

Fig. 1. Effect of neurotropin on oxaliplatin-induced mechanical allodynia in von Frey test (A) and cold hyperalgesia in acetone test (B) in rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks. Neurotropin (200 NU/kg) was administered p.o. 3 times a week for 4 weeks. Both tests were performed before the first drug administration and on Days 5, 8, 12, 15, 19, 22, 26, 29, 33, 40, 47 and 54. Values are expressed as the mean ± SEM. $^{**}P < 0.01$ compared with vehicle. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with oxaliplatin alone. The number of animals is shown in each parenthesis.

3.2. Rat sciatic nerve and DRG neurons

No histological abnormalities in sciatic nerve were observed in vehicle-, oxaliplatin- or sodium oxalate-treated rats on Day 5 (Fig. 2). Oxaliplatin (4 mg/kg, i.p.) caused the decrease in the density of myelinated fibers and the degeneration of myelinated fibers in rat sciatic nerve on Day 25. These histological changes were not observed in the tissue of rat treated with co-administration of oxaliplatin and neurotropin.

In DRG neurons, TUNEL-positive cells were hardly observed in the vehicle-, oxaliplatin- or sodium oxalate-treated rats on Day 5 (Fig. 3). Also, TUNEL-positive cells were hardly observed in the vehicle-treated rats on Day 25. Oxaliplatin markedly increased the TUNEL-positive cells on Day 25. Neurotropin had no effect on the oxaliplatin-induced increase of the TUNEL-positive cells.

3.3. Cultured PC12 and DRG cells

The exposure to oxaliplatin (0.3–30 μM) for 168 h dose-dependently shortened the length of neurites in cultured PC12 cells (0.3 μM, $P < 0.05$; 1–30 μM, $P < 0.01$, Fig. 4A). On the other hand, the exposure to sodium oxalate (0.3–30 μM) for 168 h had no effect on the length of neurites (Fig. 4B). The exposure to

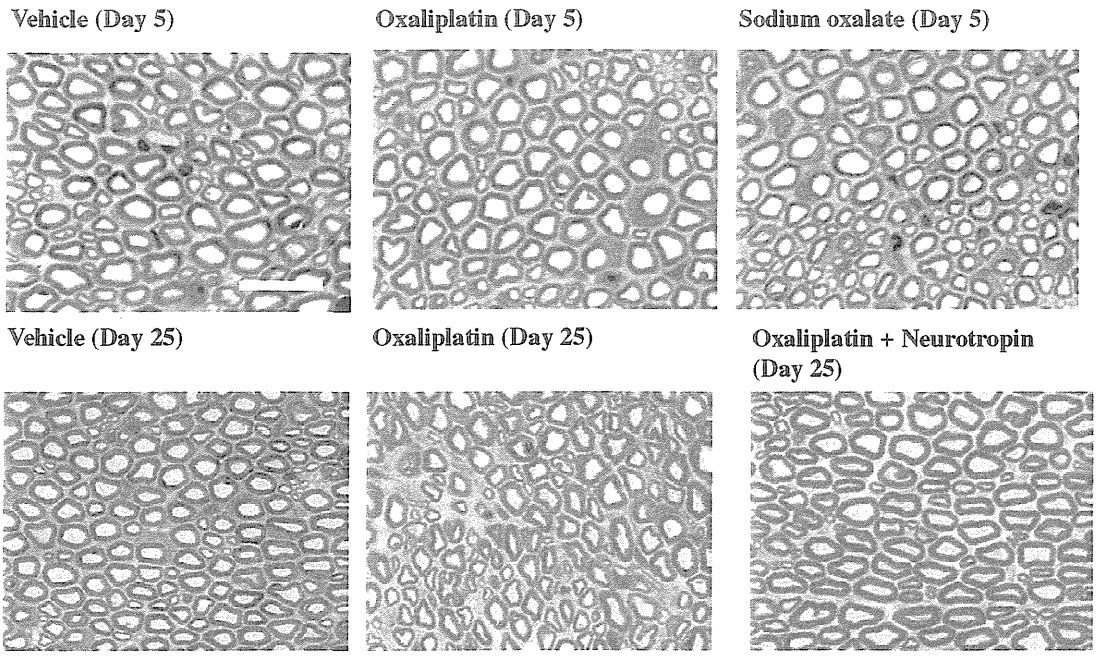

Fig. 2. Effect of neurotropin on histological change induced by oxaliplatin in rat sciatic nerve. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks. Sodium oxalate (1.3 mg/kg, i.p.) was administered twice on Days 1 and 2. Neurotropin (200 NU/kg, p.o.) was administered 3 times a week for 4 weeks. On Day 5 or 25, the sciatic nerve was harvested, and samples were stained with toluidine blue. Photographs were originally magnified 1600×. Scale bar 20 μm.

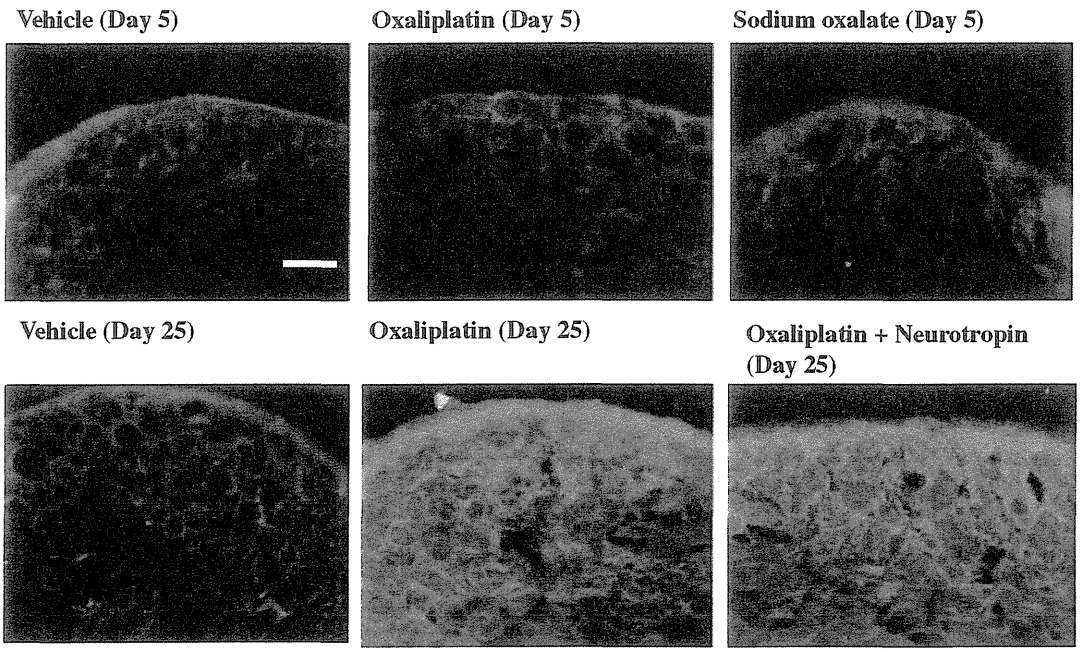

Fig. 3. Effect of neurotropin on cell injury induced by oxaliplatin in DRG neurons. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks. Sodium oxalate (1.3 mg/kg, i.p.) was administered twice on Days 1 and 2. Neurotropin (200 NU/kg, p.o.) was administered three times a week for 4 weeks. On Day 5 or 25, the L5 DRG was harvested, and samples were stained terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. Photographs were originally magnified 400×. Scale bar 50 μm.

oxaliplatin (3 μM) for 168 h also significantly shortened the length of neurites in DRG cells ($P < 0.01$, Fig. 5B). The co-exposure to neurotropin (0.001–0.03 NU/mL) for 168 h significantly extended the length of neurites compared with the oxaliplatin-treated group in the dose-dependent manner in both cultured PC12 and DRG cells (PC12: 0.003, 0.01 and 0.03 NU/mL, $P < 0.01$; DRG: 0.01 and 0.03 NU/mL, $P < 0.01$, Fig. 5).

The exposure to oxaliplatin (3–30 μM) for 168 h dose-dependently decreased the cell viability in DRG cells (3 μM, $P < 0.05$; 10, 30 μM, $P < 0.01$, Fig. 6A). On the other hand, the exposure to sodium oxalate (0.3–30 μM) for 168 h had no effect on the cell viability (Fig. 6B). The co-exposures to neurotropin (0.001–0.03 NU/mL) for 168 h had no effect on the oxaliplatin-induced decrease of the cell viability (Fig. 7).

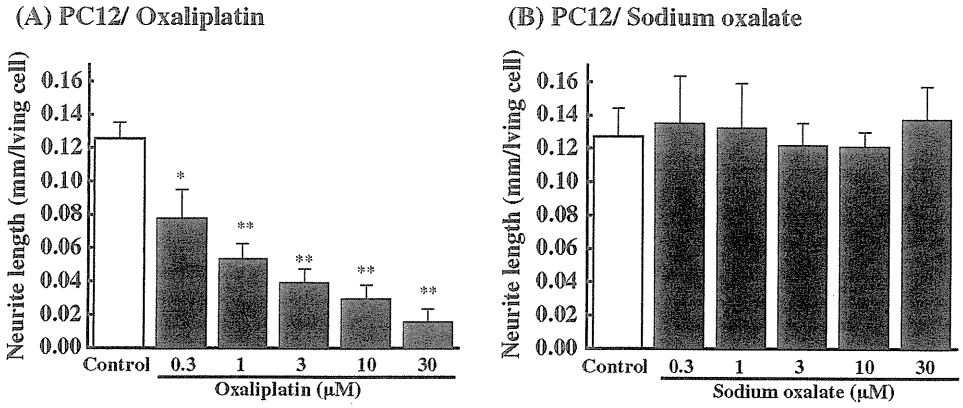

Fig. 4. Effects of oxaliplatin (A) and sodium oxalate (B) on neurite outgrowth in cultured PC12 cells. PC12 cells were incubated with oxaliplatin (0.3, 1, 3, 10 or 30 μM) or sodium oxalate (0.3, 1, 3, 10 or 30 μM) for 168 h. The neurite lengths were measured using image analysis software (Image J 1.36). Results are expressed as the mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

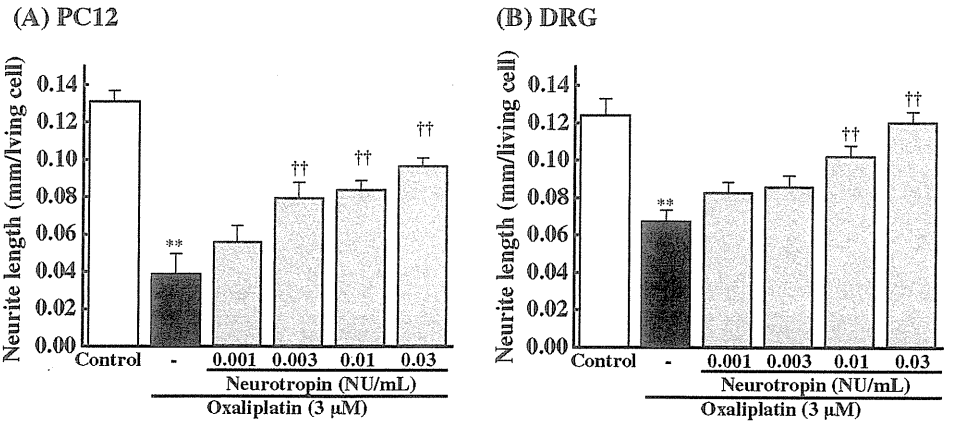

Fig. 5. Effect of neurotropin on neurite degeneration induced by oxaliplatin in cultured PC12 (A) and DRG (B) cells. Both cells were incubated with oxaliplatin (3 μM) for 168 h in the presence or absence of neurotropin (0.001–0.03 NU/mL). The neurite lengths were measured using image analysis software (Image J 1.36). Results are expressed as the mean ± SEM ($n = 8$). $^{**}P < 0.01$ compared with control, $^{\dagger\dagger}P < 0.01$ compared with oxaliplatin alone.

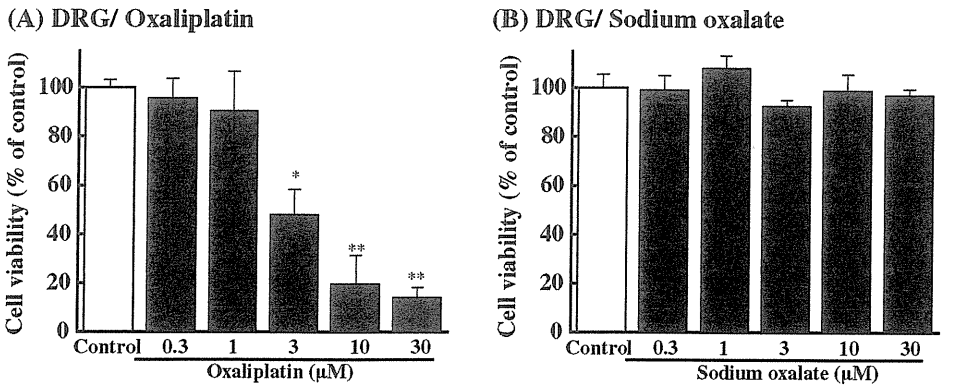

Fig. 6. Effects of oxaliplatin (A) and sodium oxalate (B) on cell viability in cultured DRG cells. DRG cells were incubated with oxaliplatin (0.3, 1, 3, 10 or 30 μM) or sodium oxalate (0.3, 1, 3, 10 or 30 μM) for 168 h. The cell viabilities were measured using WST-8 assay. Results are expressed as the mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

4. Discussion

In the present study, oxaliplatin caused cold hyperalgesia from the early phase and mechanical allodynia in the late phase, consistently with our previous report (Sakurai et al., 2009). These oxaliplatin-induced neuropathies were gradually recovered after the end of oxaliplatin administration. Clinically, the reversibility of sensory neurotoxicity is observed in patients treated with oxaliplatin (de Gramont et al., 2000). Moreover, in this study, the repeated administration of neurotropin relieved the oxaliplatin-induced mechanical allodynia in the von Frey test. On the other hand, neurotropin was almost ineffective against the oxaliplatin-induced cold hyperalgesia in the acetone test. Therefore, neurotropin may be useful for treatment of the oxaliplatin-induced chronic peripheral neuropathy.

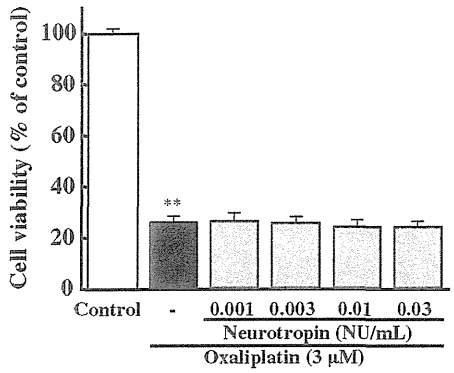

Fig. 7. Effect of neurotropin on cell injury induced by oxaliplatin in cultured DRG cells. The DRG cells were incubated with oxaliplatin (3 μM) for 168 h in the presence or absence of neurotropin (0.001–0.03 NU/mL). The cell viabilities were measured using WST-8 assay. Results are expressed as the mean ± SEM ($n = 8$). $^{**}P < 0.01$ compared with control.

In the present study, no histological abnormalities in sciatic nerve and TUNEL-positive apoptotic cell in L5 DRG were observed in oxaliplatin- or oxalate-treated rats on Day 5, although oxaliplatin caused cold hyperalgesia in the acetone test on that day. We previously reported that the oxaliplatin- and oxalate-induced hyperalgesia are completely prevented by the pre-administration of Ca^{2+} and Mg^{2+} before oxaliplatin or oxalate (Sakurai et al., 2009). Oxalate is well known as a chelator of both Ca^{2+} and Mg^{2+}. Some in vitro studies demonstrated that oxaliplatin modulates the voltage-gated Na^{+} and K^{+} channels (Adelsberger et al., 2000; Benoit et al., 2006; Kagiava et al., 2008) and that oxalate blockes the voltage-gated Na^{+} channel (Grolleau et al., 2001). Moreover, the effect of oxaliplatin on these channels has been thought to be involved in acute neuropathy (Boughattas et al., 1994; Kiernan, 2007; Saif and Reardon, 2005). Taken together with these findings, it is unlikely that the axonal degeneration in rat sciatic nerve and cell injury in DRG cells are involved in the oxaliplatin-induced cold hyperalgesia. Rather the oxaliplatin-induced cold hyperalgesia may be due to chelating of Ca^{2+} and Mg^{2+} by oxalate.

Oxaliplatin (4 mg/kg, i.p.) caused the degeneration and the decrease in the density of myelinated fibers in rat sciatic nerve on Day 25. These histological changes were not observed in the tissue of rats treated with co-administration of oxaliplatin and neurotropin. In pain behavior, neurotropin relieved the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia. Therefore, the protective effect of neurotropin on the axonal degeneration may partially contribute to the relief of the oxaliplatin-induced mechanical allodynia.

On the other hand, neurotropin had no effect on the oxaliplatin-induced increase of the TUNEL-positive cells in rat DRG neurons on Day 25. The oxaliplatin-induced cell death is completely protected by z-VAD-fmk, a caspase inhibitor, in DRG neurons (Ta et al., 2006), indicating to the involvement of apoptosis. Hence, neurotropin cannot protect the cell injury including apoptosis. We previously reported that $Pt(dach)Cl_2$ induces the mechanical allodynia in the late phase, but does not induce the cold hyperalgesia/allodynia (Sakurai et al., 2009). Several symptoms of oxaliplatin-induced chronic neuropathy are similar to those of cisplatin-induced chronic neuropathy (Pasetto et al., 2006). Oxaliplatin causes the damage of the cell bodies (Cavaletti et al., 2001; McKeage et al., 2001; Scuteri et al., 2009), the alterations in nucleus (Cavaletti et al., 2001) and nucleolus (Holmes et al., 1998; McKeage et al., 2001) and selective atrophy of subpopulation of DRG neurons (Jamieson et al., 2005) in animal models. Similarly, other platinum drugs such as cisplatin cause the damage of the cell bodies and the alterations of nucleolus (Holmes et al., 1998; McKeage et al., 2001). Moreover, oxaliplatin induces the cell death (Donzelli et al., 2004; Luo et al., 1999; Ta et al., 2006) and the inhibition of neurite outgrowth (Luo et al., 1999; Ta et al., 2006) in neuronal cells. The neurotoxicity of oxaliplatin and cisplatin for the DRG neurons correlates with platinum–DNA bindings (Ta et al., 2006). Taken together, $Pt(dach)Cl_2$ may be involved in the mechanical allodynia but not cold hyperalgesia/allodynia in the oxaliplatin-induced neuropathy, and the oxaliplatin-induced mechanical allodynia may be due to the neurotoxicity of platinum. In the present study, neurotropin is ineffective against the oxaliplatin-induced cell injury in rat DRG neurons. Therefore, the preventive effect of neurotropin on the oxaliplatin-induced mechanical allodynia might be not complete.

Ta et al. reported that oxaliplatin inhibits the neurite outgrowth and decreases the cell survival, whilst sodium oxalate does not affect the neurite outgrowth and cell survival in DRG neurons (2006). In the present study oxaliplatin shortened the length of neurites in both cultured PC12 and DRG cells and decreased the cell viability in DRG cells, whereas sodium oxalate did not affect the neurite length in PC12 cells. Hence, it is unlikely that oxalate is involved in the neurodegeneration induced by oxaliplatin. Moreover, we found that neurotropin reversed the oxaliplatin-induced neurite shortening in both cultured PC12 and DRG cells. On the other hand, neurotropin had no effect on the oxaliplatin-induced decrease of the cell viability. These results suggest that neurotropin ameliorates the neurodegeneration but not cell death induced by oxaliplatin. This hypothesis is confirmed from histological results in the tissue of rats treated with co-administration of oxaliplatin and neurotropin.

Nerve injury causes neuropathic pain via many molecular signaling including over expression of NGF (Pezet and McMahon, 2006), ion channels as voltage-gated sodium channels (Dib-Hajj et al. 2009), calcium channel alpha 2 delta subunit (Luo et al. 2001), and activation of microglia via P2X4 (Inoue and Tsuda, 2009; Tsuda et al. 2003). Especially, NGF is known to be involved in the release and upregulation of neurotransmitters such as substance P, calcitonin gene-related peptide (CGRP) and brain-derived neurotrophic factor (BDNF), and sensitization and/or upreguration of ion channels and receptors including transient receptor potential cation channel V1 (TRPV1), voltage-gated sodium channel (Nav1.8/SCN10A), P2X3, acid-sensing ion channel 3 (ASIC3) (Watson et al., 2008). These molecular mechanisms might be involved in the oxaliplatin-induced mechanical allodynia.

On Day 5, neurotropin significantly attenuated the oxaliplatin-induced cold hyperalgesia in the acetone test. Previous reports indicated single administration of neurotropin has a transient analgesic effect via activation of descending pain inhibitory system in animal pain models (Kawamura et al., 1998; Okazaki et al., 2008). Therefore, this effect might be involved in the anti-cold hyperalgesia action by neurotropin on Day 5.

In conclusion, the present results suggest that repeated administration of neurotropin relieves the oxaliplatin-induced mechanical allodynia by inhibiting the axonal degeneration. Therefore, neurotropin may be useful for the treatment of oxaliplatin-induced neuropathy.

Acknowledgements

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MOLECULAR PAIN

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L type Ca^{2+} channel blockers prevent oxaliplatin-induced cold hyperalgesia and TRPM8 overexpression in rats

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Abstract

Background: Oxaliplatin is an important drug used in the treatment of colorectal cancer. However, it frequently causes severe acute and chronic peripheral neuropathies. We recently reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats, and that oxalate derived from oxaliplatin is involved in the cold hyperalgesia. In the present study, we examined the effects of Ca^{2+} channel blockers on oxaliplatin-induced cold hyperalgesia in rats.

Methods: Cold hyperalgesia was assessed by the acetone test. Oxaliplatin (4 mg/kg), sodium oxalate (1.3 mg/kg) or vehicle was injected i.p. on days 1 and 2. Ca^{2+} (diltiazem, nifedipine and ethosuximide) and Na^{+} (mexiletine) channel blockers were administered p.o. simultaneously with oxaliplatin or oxalate on days 1 and 2.

Results: Oxaliplatin (4 mg/kg) induced cold hyperalgesia and increased in the transient receptor potential melastatin 8 (TRPM8) mRNA levels in the dorsal root ganglia (DRG). Furthermore, oxalate (1.3 mg/kg) significantly induced the increase in TRPM8 protein in the DRG. Treatment with oxaliplatin and oxalate (500 μM for each) also increased the TRPM8 mRNA levels and induced Ca^{2+} influx and nuclear factor of activated T-cell (NFAT) nuclear translocation in cultured DRG cells. These changes induced by oxalate were inhibited by nifedipine, diltiazem and mexiletine. Interestingly, co-administration with nifedipine, diltiazem or mexiletine prevented the oxaliplatin-induced cold hyperalgesia and increase in the TRPM8 mRNA levels in the DRG.

Conclusions: These data suggest that the L type Ca^{2+} channels/NFAT/TRPM8 pathway is a downstream mediator for oxaliplatin-induced cold hyperalgesia, and that Ca^{2+} channel blockers have prophylactic potential for acute neuropathy.

Background

Oxaliplatin, a platinum-based chemotherapeutic agent, is widely used for treatment of colorectal cancer. However, oxaliplatin frequently causes severe acute and chronic peripheral neuropathies. Acute neuropathy is peculiar to oxaliplatin and includes acral paresthesias enhanced by exposure to cold [1-4]; the acute neuropathy is not attributed to morphological damage to the nerve [5,6]. On the other hand, the chronic neuropathy is characterized by loss of sensory and motor function after long-term oxaliplatin treatment, and it is similar to cisplatin-induced neurological symptoms [4]. Recently, we reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats [7]. Oxaliplatin is metabolized to oxalate and dichloro (1,2-diaminocyclohexane)platinum [Pt(dach)Cl_2] [8]. We reported that oxalate and platinum metabolites were involved in the cold hyperalgesia and mechanical allodynia, respectively, and that intravenous pre-administration of Ca^{2+} or Mg^{2+} prevented the cold hyperalgesia but not mechanical allodynia in rats [7].

Oxaliplatin-induced acute neuropathy is termed a 'channelopathy', as oxaliplatin and oxalate were shown to modulate voltage-gated Na^{+} and K^{+} channels in

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several types of neurons [9-12]. For example, oxaliplatin increases the amplitude and duration of compound action potentials interacting with voltage-gated Na^+ channels in rat sensory neurons [9]. Furthermore, oxaliplatin prolongs the duration of the A-fiber compound action potential related to K^+ channels [12]. Thus, the effect of oxaliplatin on Na^+ and K^+ channels is thought to be involved in acute neuropathy [13].

Transient receptor potential (TRP) melastatin 8 (TRPM8), an ion channel that belongs to the TRP family, is activated by innocuous cold (< 25°C) or menthol [14,15]. Recently, an increase in TRPM8 mRNA levels was reported to be involved in the oxaliplatin-induced cold hyperalgesia in mice [16]. However, the molecular mechanisms mediating the acute neuropathy remain unclear. In the present study, we investigated the involvement of voltage-gated Ca^{2+} channels, nuclear factor of activated T-cell (NFAT) and TRPM8 in the oxaliplatin-induced cold hyperalgesia, as up-regulation of TRP channel 1 (TRPC1) expression is induced by store-operated calcium (SOC) channel/NFAT, a transcription factor regulated by the calcium signaling pathway [17]. Furthermore, we investigated the effects of Ca^{2+} channel blockers on oxaliplatin-induced cold hyperalgesia in rats.

Methods

Drugs and chemicals

Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo, Japan). Sodium oxalate was provided by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mexiletine hydrochloride, nifedipine, diltiazem hydrochloride and ethosuximide were purchased from Sigma-Aldrich, Co. (MO, USA). Vivit was purchased from Calbiochem (Darmstadt, Germany).

Animals

Male Sprague-Dawley rats weighing 200-250 g (Kyudo Co., Saga, Japan) were used. Rats were housed in groups of four to five per cage with lights on from 07:00 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 the International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues guidelines for animal research [18].

Behavioral test

Cold hyperalgesia was assessed by the acetone test. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor, and were allowed to habituate for 30 min prior to testing. Fifty microliters of acetone (Wako Pure Chemical Industries, Ltd.) was sprayed onto the plantar skin of each hind paw 3 times with a Micro Sprayer® (Penn Century Inc., PA, USA), and the number of withdrawal response was counted for 40 s from the start of the acetone spray. Acetone tests were performed on days 0 (pre), 3, 5, 8 and 15. Oxaliplatin and sodium oxalate were dissolved in 5% glucose solution. Oxaliplatin (4 mg/kg), sodium oxalate (1.3 mg/kg) or vehicle was injected i.p. on days 1 and 2. The vehicle-treated rats were injected with 5% glucose solution. Mexiletine hydrochloride, diltiazem and ethosuximide were dissolved in 5% saline. Nifedipine was suspended in 1% carboxymethylcellulose sodium solution. Mexiletine, diltiazem, nifedipine and ethosuximide were administered p.o. simultaneously with oxaliplatin or oxalate on days 1 and 2.

Cell cultures

Male Sprague-Dawley rats (6 weeks old, Kyudo Co.) were anesthetized with sodium pentobarbital, and the L 4-6 dorsal root ganglia (DRG) cells were removed and primary cultured. Ganglia were incubated with 0.125% (w/v) collagenase type 1 (Worthington Biochemical Corp., NJ, USA) at 37°C for 90 min followed by incubation with 0.25% (w/v) trypsin-EDTA (Gibco BRL, CA, USA) for 30 min. DRG cells were grown in Dulbecco's modified Eagle's medium (MP Biomedicals, Inc., CA, USA) with 2 mM L-glutamine and 10% FBS. The cells were cultured at 37°C in air supplemented with 5% CO_2 under humidified conditions. Oxaliplatin, oxalate, mexiletine, diltiazem and ethosuximide were dissolved in medium. Nifedipine and vivit were dissolved in 0.2% DMSO.

Measurement of intracellular Ca^{2+} level

Cells were loaded with 5 μg/mL of Fura-2/AM (Dojindo Lab., Kumamoto, Japan) and then incubated for 1 h at 37°C in HEPES buffer. The Fura-2/AM-loaded cells were washed and placed in HEPES buffer. The intracellular Ca^{2+} levels were determined by emission fluorescence at 510 nm with excitation at 340 nm and 380 nm, using FlexStation3 (Molecular Devices, Inc., CA, USA).

Immunostaining of NFATc4

Immunofluorescent staining for NFATc4 was performed using a rabbit monoclonal antibody (Cell Signaling Technology, Inc., MA, USA). Briefly, cells were cultured on cover slips, and the cover slips then rinsed with ice-cold phosphate-buffered saline and fixed with 4% (w/v) ice-cold paraformaldehyde for 30 min at -20°C. The NFATc4 antibody was diluted (1:100) with phosphate-buffered saline (PBS) containing 5% (w/v) bovine serum albumin and 0.1% Triton X-100. Cells were incubated with diluted antibody solution overnight in a humidified

chamber at 4°C. After washing with PBS, cover slips were incubated at room temperature for 1 h with goat anti-rabbit IgG (1:500 dilution in PBS) that was conjugated with Alexa Fluor® 488 (Cell Signaling Technology, Inc.). The nucleus was stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; Dojindo Lab.). NFATc4 and nuclear staining were visualized with a fluorescence microscope (BX51; Olympus Corp., Tokyo, Japan). The nuclear translocation of NFATc4 was calculated by comparing the ratio of nuclear NFATc4 immunofluorescence/total NFATc4 immunofluorescence using analysis software (Image J 1.36; Wayne Rasband, National Institutes of Health, MD, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

mRNA was isolated from L4-6 DRG using PolyATtract® System 1000 (Promega, Corp., WI, USA). cDNA was synthesized with PrimeScript® 1st strand cDNA Synthesis Kit (TaKaRa Bio, Inc., Shiga, Japan). PCR was performed with Gene Taq (Nippon Gene, Co., Ltd., Tokyo, Japan). The oligonucleotide primers for TRPM8 were designed based on the sequences described by Ta and colleagues [19]. The sequences of PCR primers were as follows: TRPM8, 5'-GCC CAG TGA TGT GGA CAG TA-3' (sense), 5'-GGA CTC ATT TCC CGA GAA GG-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, Co.). Reactions were run for 35 cycles with 95°C denaturing cycle (30 s), 62°C annealing cycle (1 min) and 72°C extension cycle (20 s) for TRPM8, 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. PCR products were resolved by electrophoresis on a 4% agarose gel, and the DNA was visualized by staining with ethidium bromide under ultraviolet irradiation. The intensities of the PCR products were semi-quantified densitometrically using Alpha Imager 2200 (Cell Biosciences, Inc., California, USA).

Western blotting

The L4-6 DRG was quickly removed on day 5. 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 4% 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 TRPM8 antibody (Abcam, MA, USA) and then incubated for 1 h with anti-rabbit IgG horseradish peroxidase (Jackson Immuno Research Laboratories, Inc., PA, USA). The immunoreactivity was detected using Enhanced Chemiluminescence (Perkin Elmer, Massachusetts, USA).

Statistical analyses

Data are expressed as the mean ± SEM. Data were analyzed by the Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test to determine differences between the groups. A probability level of $p < 0.05$ was accepted as statistically significant.

Results

Oxaliplatin increases cold hyperalgesia and TRPM8 expression in the DRG in rats

Administration of oxaliplatin (4 mg/kg, p.o., on days 1 and 2) significantly increased the number of withdrawal responses to cold stimulation by acetone spray in rats (Figure 1A, days 3, 5 and 8: $p < 0.01$). This increase in withdrawal response had disappeared on day 15. On day 5, TRPM8 mRNA levels in the L4-6 DRG of oxaliplatin-treated rats markedly increased as compared with those of vehicle-treated rats (Figure 1B, $p < 0.01$). Also, oxalate treatment significantly induced the increase in TRPM8 protein in the L4-6 DRG (Figure 1C, $p < 0.05$).

Oxaliplatin and oxalate increase the TRPM8 mRNA levels in primary cultured DRG cells

Treatment with either oxaliplatin (Figure 1D) or oxalate (Figure 1E) for 12 h markedly increased the TRPM8 mRNA levels in primary cultured DRG cells ($p < 0.05$ for both).

Oxaliplatin and oxalate increase the intracellular Ca^{2+} levels in primary cultured DRG cells

Oxaliplatin and oxalate (100-500 μM) induced dose-dependent increases in intracellular Ca^{2+} levels in cultured DRG cells (Figure 2A, B). The percentages of DRG neurons that responded to oxaliplatin and oxalate were 69.2% and 64.0%, respectively. Nifedipine (30 μM), an L type Ca^{2+} channel blocker, and diltiazem (30 μM), an L/T type Ca^{2+} channel blocker, inhibited the increase in intracellular Ca^{2+} levels induced by oxalate (500 μM) (Figure 2C, D). Mexiletine, a Na^+ channel blocker, also dose-dependently inhibited the oxalate-induced increase in intracellular Ca^{2+} levels (Figure 2F). By contrast, ethosuximide (1 mM), a T type Ca^{2+} channel blocker, only weakly attenuated the oxalate-induced increase in intracellular Ca^{2+} levels (Figure 2E).

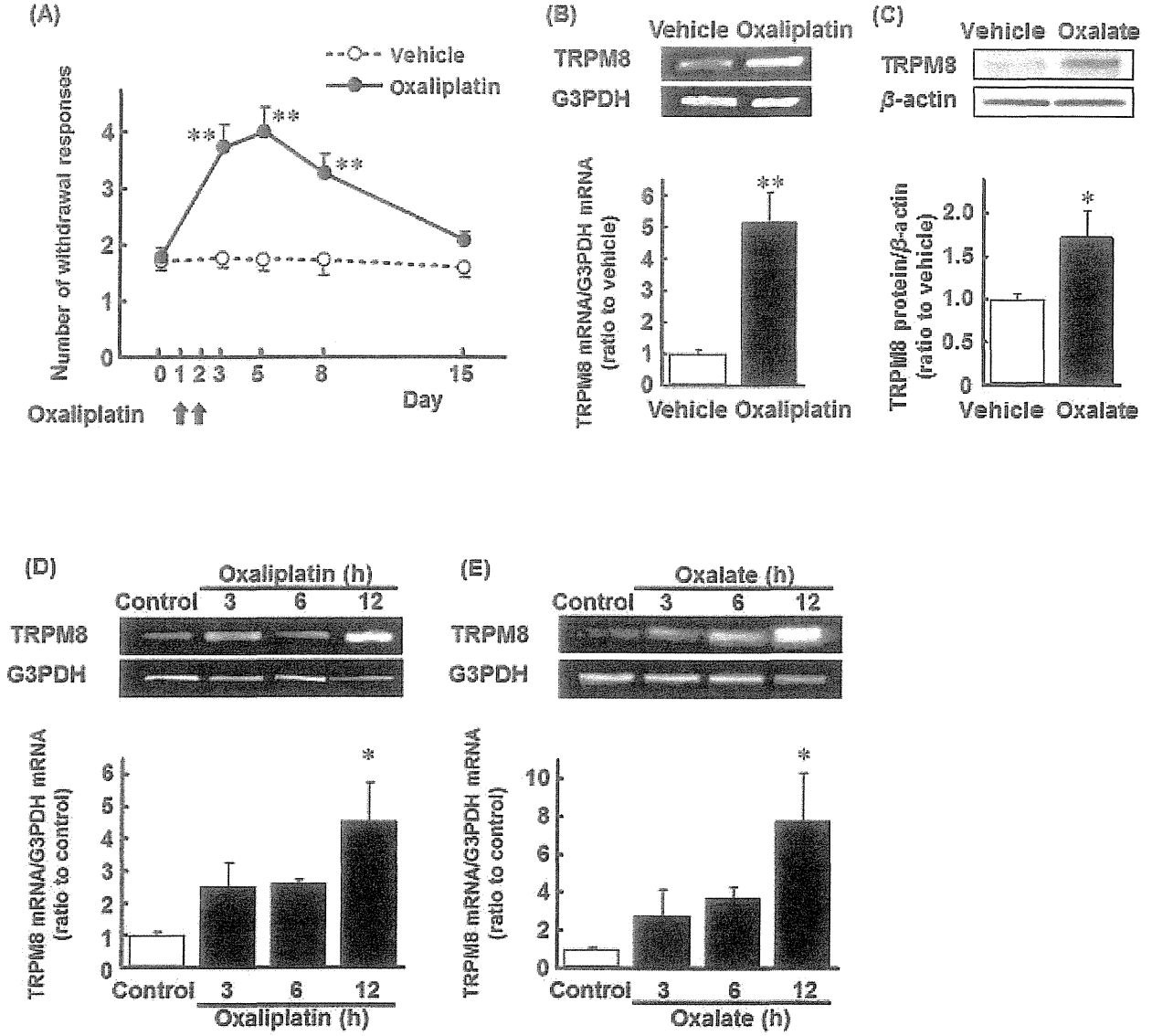

Figure 1 The incidence of cold hyperalgesia (A) and expression of TRPM8 (B-E) following oxaliplatin or sodium oxalate treatment. Oxaliplatin (4 mg/kg) or sodium oxalate (1.3 mg/kg) was administered i.p. on days 1 and 2. **A**: The acetone test was performed on days 0, 3, 5, 8 and 15. **B**: On day 5 the rat L4-6 DRG treated with oxaliplatin was harvested and the mRNA expression of TRPM8 and G3PDH were determined by PCR. **C**: On day 5 the rat L4-6 DRG treated with sodium oxalate was harvested and the protein of TRPM8 and β-actin were determined by Western boltting. **D, E**: 500 μM of oxaliplatin (D) or sodium oxalate (E) was administered to cultured DRG cells for 3, 6 or 12 h. mRNA expression of TRPM8 and G3PDH was determined by PCR. Values are expressed as the mean ± SEM of 4-6 animals (A, B) or 4-6 wells (C, D). $*p < 0.05$, $**p < 0.01$ compared with vehicle or control group.

Oxaliplatin and oxalate induce NFAT nuclear translocation in primary cultured DRG cells

Treatment with oxaliplatin (500 μM) for 6 h induced NFAT nuclear translocation (Figure 3A, B, 6 h: $p < 0.01$). Similarly, 500 μM oxalate caused NFAT nuclear translocation (Figure 3C, D, 6 h: $p < 0.01$). Mexiletine (1 mM), nifedipine (30 μM) and diltiazem (30 μM) completely blocked the oxalate-induced NFAT nuclear translocation (500 μM) (Figure 3E, F, $p < 0.01$). Similarly, vivit (2 μM), a selective NFAT inhibitor, completely blocked the oxalate-induced NFAT nuclear translocation (Figure 3E, F, $p < 0.01$).

Ca^{2+} and Na^{+} channel blockers inhibit the oxalate-induced increase of TRPM8 mRNA levels in cultured DRG cells

Mexiletine (1 mM), nifedipine (30 μM) and diltiazem (30 μM) reversed the increase in TRPM8 mRNA levels induced by oxalate (500 μM, 12 h) (Figure 4, mexiletine