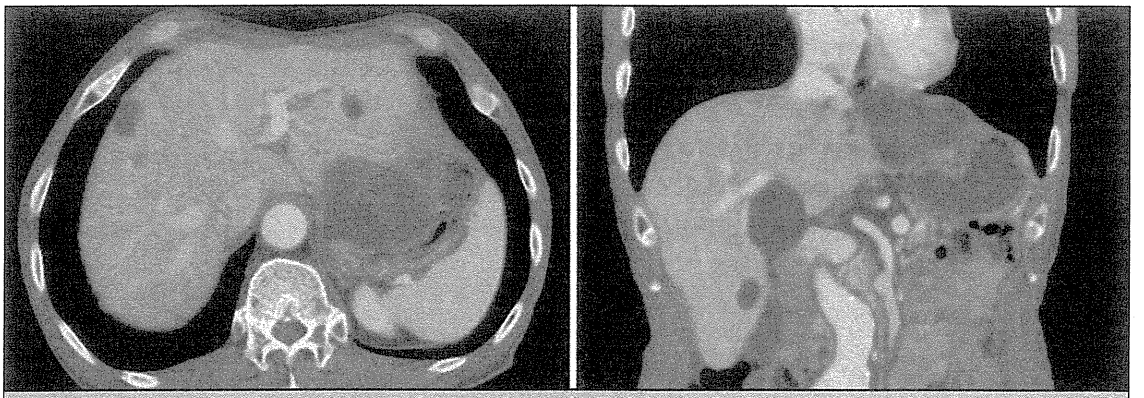

Fig. 3 Three months after treatment with imatinib mesylate, abdominal enhanced CT showed reduction in tumor size.

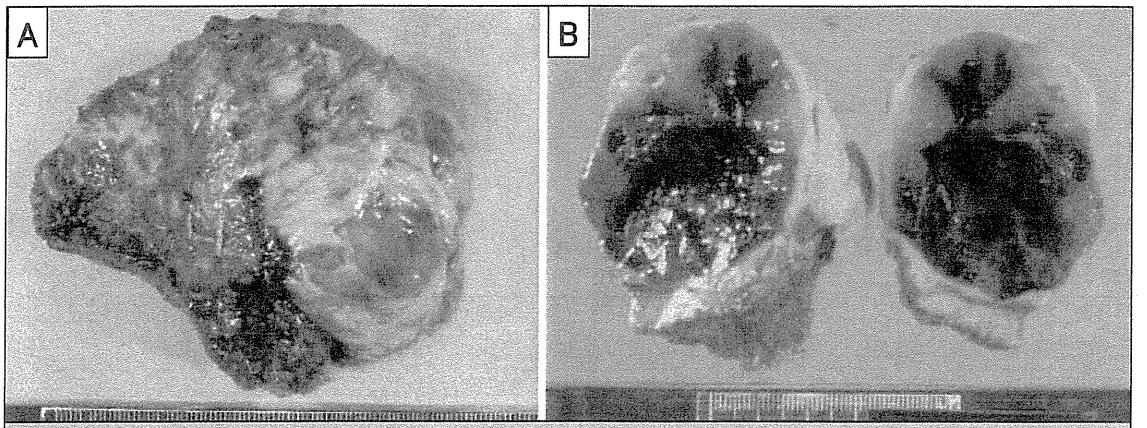

Fig. 4 Macroscopic appearance of the tumor (A. B). A: The resected preparation, showing a 7.0×6.0 cm tumor. B: The cut surface of the tumor is dark reddish like an old hematoma.

の横隔膜の合併切除が必要であると思われ，侵襲が大きいと考えられた．術前化学療法を行う方針とし，イマチニブ（400mg/day）を約2か月間投与した．2009年2月の腹部造影CTでは腫瘍は縮小し，横隔膜への浸潤範囲も縮小していた（**Fig. 3**）．この時点で切除可能と判断し，手術目的に入院となった．

現症：体温36.7℃，血圧124/80mmHg，脈拍64/分，腹部には腫瘤を触知せず，正中部に前回手術痕を認めた．

血液生化学検査：異常所見は認めず，腫瘍マーカーの上昇もみられなかった．

腹部造影CT所見：腫瘍は7.5×6.0cmと縮小を認め，腫瘍内の造影効果も減弱していた（**Fig. 3**）．横隔膜への浸潤範囲も縮小していた．画像上PR，切除可能と判断した．

イマチニブの効果はPRであったが，今後の投与継続による副作用や耐性の可能性も考えられたため切除する方針とした．

手術所見：腫瘍は肝外側区，食道胃吻合部付近を主座としていた．肉眼的に横隔膜への浸潤は認めなかった．残胃部分切除術，肝外側区域切除術を施行した．

切除標本肉眼所見：腫瘍は被膜を有する7.0×6.0cmの充実性の腫瘤であった．割面では全体に暗赤色調を呈しており，典型的なGISTに見られるような黄白色調ではなかった（**Fig. 4**）．

病理組織学的検査所見：GIST細胞は認めず，血管腫様組織が主体であった．viable cellは認めず，pCRと判定された（**Fig. 5**）．また遺伝子解析は行わなかった．

術後経過：術後にイマチニブの投与は行わず，外来にて経過観察中であるが，術後1年経過時点で再発は認めていない．

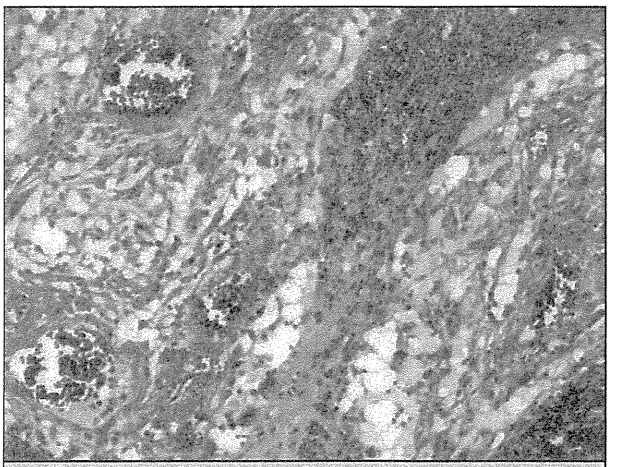

Fig. 5 Histopathological findings: Most of the tumor was replaced by angiomatoid tissue, and no viable tumor cells were detected.

考　察

本邦での GIST 診療ガイドライン[2]によると，再発・転移 GIST に対する治療の原則はイマチニブ投与である．再発 GIST に対しては，以前は再手術しか有効な治療法がなく，切除不能であった場合の予後は非常に不良であった．欧米で行われたイマチニブの第 II 相臨床試験[3]によると，奏効率で 53.7%，SD を含めた病勢コントロール率で 81.6% と，高い抗腫瘍効果が報告されている．また同試験の遺伝子解析では，イマチニブの抗腫瘍効果は c-kit 遺伝子変異部位により異なることが報告されており，エクソン 11 に変異がみられる症例では奏効率は 84% と最も高く，エクソン 9 の変異では 48% であった．

しかしながら，国内外の臨床試験の結果でもイマチニブ治療単独での CR 例はほとんどなく，イマチニブの投与により腫瘍のサイズが縮小しても，病理組織学的には viable な腫瘍細胞が一部に残存しているものと考えられている[4]．イマチニブの休薬により，早期の増悪が報告されており，病勢のコントロールにはイマチニブを可能なかぎり長期に継続することが必要である．その一方で，イマチニブ投与による副作用や二次耐性の出現により，イマチニブの継続が困難となる場合も少なくない．

近年では，再発 GIST に対しイマチニブを投与後に再切除を行い，良好な結果を得ている報告も散見される[4)~6)]．NCCN の GIST 治療ガイドライン[7]では，再発 GIST に対する外科的治療の原則として，イマチニブに反応のある GIST や，イマチニブで進行が停止し安定化した GIST，あるいは効果がなく進行する GIST で残存腫瘍が完全切除可能な場合には，外科的手術も適応となるとしている．

Rutkowski ら[8]や Gronchi ら[9]は，切除不能，転移再発 GIST に対するイマチニブ投与後の腫瘍切除について，イマチニブ奏効中の腫瘍切除が予後良好であり，腫瘍がイマチニブ耐性を獲得し病勢コントロールが得られない状態での手術では，切除率，生存率がともに低下し，予後不良になると報告している．

本症例も，局所再発を来した胃 GIST に対してイマチニブの投与を行い，病勢のコントロールを行ったうえで手術を施行し，残存腫瘍の完全切除を施行することができた．切除標本の病理組織学的検討では，腫瘍部には血管腫様の組織がみられるのみで，GIST の viable cell は認めず pCR と判断した．切除標本上 GIST 細胞は認められなかったが，イマチニブ投与により画像上縮小が得られたことから本症例は GIST の再発に矛盾しないと考えた．

イマチニブは腫瘍細胞の増殖を抑制し，アポトーシスを誘導するため，腫瘍細胞は嚢胞化や硝子様変性を起こすとされている[4]．本症例ではそのような変化は認めなかったが，血流の豊富な腫瘍細胞の中で血管が増生し，イマチニブの抗腫瘍効果により腫瘍細胞が消退，その結果栄養血管のみが残り，血管腫

Table 1 Reported cases of pathological complete response obtained after neoadjuvant chemotherapy by imatinib mesylate in Japan

Case	Author Year	Age Sex	Initial therapy	Recurrence	Tumor size before administration of IM (cm)	Response to treatment	Operation after administration of IM	Pathological findings
1	Abeshima[10] 2004	66 M	Administration of IM	None	Over fist size	PR	Total gastrectomy Left lobectomy of the liver	Hyaline degeneration
2	Nojiri[5] 2005	52 M	Partial gastrectomy	Local Liver	4 (Local), 7 (Liver)	SD	Proximal gastrectomy Partial hepatectomy	Hyaline degeneration
3	Koeda[6] 2007	56 M	Total gastrectomy	Spleen	4×4	PR	Splenectomy	Myxoid degeneration
4	Our case	78 M	Proximal gastrectomy	Local	8.5×7.2	PR	Partial gastrectomy Partial hepatectomy	Angiomatoid change

IM: imatinib mesylate

様の所見を呈したものと考えられた.

切除後の治療については, Rutkowski ら[8]や Gronchi ら[9]のいずれもイマチニブの投与継続が重要で, 術後できるだけ早期にイマチニブを再開することが必要であるとしている. 本症例は pCR が得られたため, 手術後のイマチニブ投与は行っていないが, 現在まで再発を認めていない.

胃 GIST に対して術前化学療法を行い切除した症例において pCR が得られることはまれである.「胃 GIST」,「メシル酸イマチニブ」をキーワードに医学中央雑誌刊行会 Web 版で 1983 年から 2010 年まで検索すると, 再発例も含めた胃 GIST に対して術前化学療法を施行後に手術を行った報告は 15 例であった. このうち切除標本で pCR を確認したのは 3 例[5)6)10)]のみであった(会議録を除く). 表に示すように自験例を含めた 4 例の検討では, 安部島ら[10]が報告した 1 例は初回治療にイマチニブを使用しているが, その他の 3 例は手術後の再発例に対してイマチニブを使用している (Table 1). 本症例では初回切除後に 1 年 9 か月間イマチニブを投与しているが, 術後約 4 年で局所再発を来したため, イマチニブの投与を再開した. 本症例は再発時の腫瘍径も大きく, 横隔膜や肝外側区への浸潤も見られたが, イマチニブによる術前化学療法が奏効し手術可能となり, 切除標本で pCR が得られた希少な症例であると考えられた.

なお, 本論文の要旨は第 71 回日本臨床外科学会 (京都) で発表した.

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CASE REPORT

A Resected Case of Local Recurrence of GIST of the Stomach in which Pathological complete Response after Neoadjuvant Chemotherapy by Imatinib Mesylate

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A 78-year-old man underwent proximal gastrectomy for gastrointestinal stromal tumor (GIST) of the stomach in November 2004.The pathological diagnose was GIST, and it was positive for KIT and CD34 immunohistochemically, indicating it was in a high risk group. Administration of imatinib mesylate at a dose of 400 mg/day was given for 21 months after surgery as adjuvant chemotherapy. In November 2000, abdominal computed tomography (CT) revealed local recurrence of GIST invading the liver and diaphragm making curative resection difficult, so administration of imatinib mesylate was restarted. Three months after reinitiating imatinib mesylate treatment,abdominal CT showed reduction in tumor size. Therefore,we judged this lesion to be resectable and performed local resection. Histopathologically, the tumor was replaced by angiomatoid change,and no viable tumor cells were detected. Pathological complete response (pCR) was obtained. This was a rare case, in which local recurrence of GIST invading the liver and diaphragm was resected after neoadjuvant chemotherapy by imatinib mesylate, and pathological complete response was obtained.

Key Words: GIST, imatinib mesylate, neoadjuvant therapy

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食道癌に対する化学療法後の手術

Esophagectomy after chemotherapy

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【ポイント】
◆ 切除不能食道癌は非常に予後不良である．
◆ 食道扁平上皮癌はほかの固形癌に比べて抗癌剤や放射線への感受性が高い．
◆ 切除困難症例を切除可能症例にするには補助療法が必要である．

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はじめに

食道癌の治療成績は徐々に向上してきている．これは，診断や治療を含めた内視鏡技術の進歩や検診の普及などで比較的早期に癌が発見される割合が増えていることや，また，周術期管理の向上や頸部・胸部・腹部3領域の徹底したリンパ節郭清を中心とした手術手技の向上が背景にある．さらに，近年では術前または術後に補助化学療法を行うことで，さらに治療成績の向上をもたらしている[1,2]．一方，食道癌はほかの消化器癌に比べて悪性度が高く，他臓器浸潤や広範なリンパ節転移を伴った高度進行食道癌で発見されることもいまだ少なくない．腫瘍自体が他臓器浸潤のある症例（T4）や所属リンパ節を介した他臓器浸潤を認める場合は，相手臓器が容易に合併切除可能な臓器に限りT3症例に準じた手術適応を決定するが，気管・気管支や大血管への浸潤が認められる場合には一般に化学療法や放射線療法もしくは化学放射線療法が優先される．しかしながら，わが国の報告においてT4食道癌の治療成績は，手術症例において5年生存率が11.9%[3]，化学放射線療法の効果を検証した第II相臨床試験（JCOG9516）においてもcomprete response（CR）率が15%，2年生存率が32%であり[4]，決して十分な治療成績とはいえない．そこで，局所進行食道癌においては，T4が解除できた時点で手術療法へ移行するか，根治的な非手術療法を継続するかの検討がなされる[5]．

本稿では，局所進行によって切除困難な食道癌の治療戦略について概説する．

局所進行食道癌に対する治療戦略

「食道癌診断・治療ガイドライン」（2007年4月版）[6]の治療アルゴリズムでは，進行食道癌stageIII（T4）・IVaに対しては化学放射線療法もしくは放射線療法，遠隔臓器転移を伴うstageIVbに対しては化学療法，放射線療法もしくは切除不能症例の治療と記載されており（図1），他臓器合併切除が可能な一部の症例を除いて初回治療として手術療法が選択されることはほとんどない．遠隔臓器転移がなく，腫瘍が気管・気管支や大動脈などの食道周囲臓器へ浸潤した症例（T4）や所属リンパ節転移を介した他臓器浸潤を認める場合は，癌が局所にとどまっていることから根治的化学放射線療法の適応となる．また，TNM分類でM1（LYM）に相当する可動性のない鎖骨上窩のリンパ節転移，もしくは腹腔動脈周囲リンパ節転移などが認められる症例も一般的に切除不能であり，かつ癌が局所（照射可能範囲）にとどまっていることから根治的化学放射線療法の適応となる．ただし，頸部食道癌では気管浸潤

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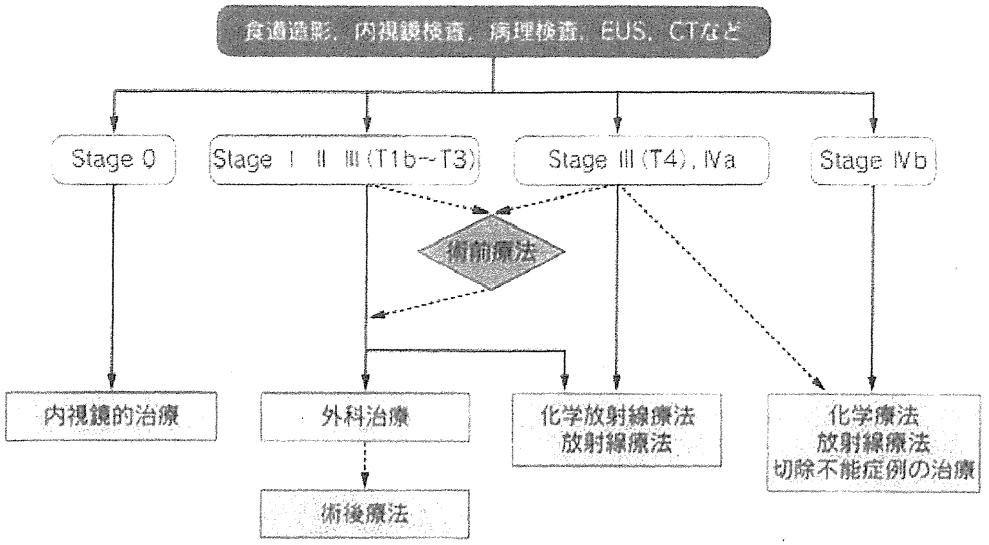

図1　食道癌治療アルゴリズム　（文献6より転載）

（T4）が認められた場合でも合併切除が可能であるため，手術療法が行われることもある．一方，腹部大動脈周囲リンパ節転移や遠隔臓器転移が認められる症例，通過障害が高度でなく頸部・胸部・腹部の3領域リンパ節転移が認められる症例に対しては全身化学療法が施行されることが一般的である．

切除困難（不能）食道癌に対する，手術を前提とした化学療法または化学放射線療法（induction therapy）後の食道切除の治療成績を検討した大規模試験は主に海外から報告されている．術前化学放射線療法＋手術と根治的化学放射線療法を比較するrandomized controlled trial（RCT）として，Stahlら[7]はcT3-4，N0-1，M0の食道扁平上皮癌患者を対象にinduction chemotherapy（5-FU＋ロイコボリン＋エトポシド＋シスプラチン）後に術前化学放射線治療（40 Gy）＋手術をする群と，根治的化学放射線治療（65 Gy以上）を施行する群の比較を行った．また，Bedenneら[8]はcT3，N0-1，M0の胸部食道癌患者を対象に化学放射線療法（5-FU＋シスプラチン＋照射30～46 Gy）後の奏効例に対して，手術群と根治照射まで化学放射線療法を継続する群とで比較試験を行っている．これらの試験の結果では，術前化学放射線が奏効した症例では化学放射線の継続が手術とほぼ同等の成績であるが，局所制御の点では術前化学放射線療法＋手術群のほうが優れているとの結果であった．

また，切除不能症例とは限らないが，術前に補助療法として化学療法あるいは化学放射線療法を行うことの有効性を検討した臨床試験が海外から報告されている[9]．術前化学療法による生存率改善効果を検討したRCTは9試験報告されており，そのうち生存率改善に寄与すると報告されたのは2編である．また，術前化学放射線療法による生存率改善効果を検討したRCTは7試験あり，そのうち生存率改善に寄与すると報告されたのは1編のみであった．しかしながら，これらの報告では手術単独での治療成績がわが国に比べて低く，頸部・胸部・腹部3領域リンパ節の徹底郭清が基本術式となっているわが国と，手術による局所制御を含めた治療効果の点で相違がみられる．また，これらのRCTでは扁平上皮癌と腺癌などの組織型の違いや，化学療法のレジメン，照射線量なども各試験で異なり，その検証に関しては慎重に判断する必要があると思われる．

一方，わが国においては，切除可能であるstage Ⅱ/Ⅲを対象とした臨床試験（JCOG9906）の結果において，根治的化学放射線療法（5-FU＋シスプラチン＋照射60 Gy）施行後の治療成績がCR率68%，3年生存率46%と報告されている[10]．この試験の結果は，手術関連死亡率が約10%とされるサルベージ手術を含んだ治療成績であり，単純にその成績を評価することはできないが，その高いCR率を考慮すると，切除困難症例に対するinduction therapyとして，照射線量の設定などの問題は含むが有用なプロトコールの1つとして挙げられると思われる．

Brain and Behavior

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Oxaliplatin-induced neurotoxicity involves TRPM8 in the mechanism of acute hypersensitivity to cold sensation

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Abstract

Oxaliplatin-induced peripheral neurotoxicity (OPN) is commonly associated with peripheral hypersensitivity to cold sensations (CS) but the mechanism is unknown. We hypothesized that the transient receptor potential melastatin 8 (TRPM8), a putative cold and menthol receptor, contributes to oxaliplatin cold hypersensitivity. To determine whether the TRPM8 is involved in acute OPN, varying concentrations of menthol were topically applied to the tongues of healthy subjects ($n = 40$) and colorectal cancer patients ($n = 36$) before and after oxaliplatin administration. The minimum concentration of menthol to evoke CS at the menthol application site was determined as the CS detection threshold (CDT). In healthy subjects, the mean CDT was 0.068. Sex and age differences were not found in the CDT. In advanced colorectal cancer patients, the mean CDT significantly decreased from 0.067% to 0.028% ($P = 0.0039$) after the first course of oxaliplatin infusions, and this marked CS occurred in patients who had grade 1 or less neurotoxicity, and grade 2 neurotoxicity, but not in those with grade 3 neurotoxicity. Further, the mean baseline CDT in oxaliplatin-treated patients was significantly higher than that of chemotherapy-naïve patients and healthy subjects (0.151% vs. 0.066%, $P = 0.0225$), suggesting that acute sensory changes may be concealed by progressive abnormalities in sensory axons in severe neurotoxicity, and that TRPM8 is subject to desensitization on repeat stimulation. Our study demonstrates the feasibility of undertaking CDT test in a clinical setting to facilitate the identification of early neurotoxicity. Moreover, our results indicate potential TRPM8 involvement in acute OPN.

Introduction

Oxaliplatin-induced peripheral neurotoxicity (OPN) is deleterious to patients both in terms of troublesome symptoms and the need to reduce or discontinue chemotherapy (Adelsberdger et al. 2000). Oxaliplatin, a third-generation platinum analog, causes a unique spectrum of acute peripheral nerve hyperexcitability that has not been observed in patients receiving other platinum chemotherapeutic agents. Conversely, chronic oxaliplatin treatment induces an axonal neuropathy that is similar to that observed with other platinum-based compounds (Lehky et al. 2004). In clinical studies, approximately 90% of oxaliplatin-treated patients experienced unique acute OPN, particularly cold-induced paresthesia that is usually triggered by cold exposure and begins in the hands or feet but sometimes occurs around the mouth or in the throat (Raymond et al. 1998a; Raymond et al. 1998b; Grothey, 2003; Ali 2010;). It is an acute transient syndrome that may begin during drug infusion or within minutes, hours, or 1–2 days after administration but is usually self-limiting, often disappearing within a few days (Gamelin et al. 2002, 2006).

Recently, a wide repertoire of sensory transduction molecules that convert external environmental stimuli into neural activity has been identified (Basbaum et al. 2009). For example, the transient receptor potential (TRP) family of ion channels are the primary detectors of thermal stimuli (Jordt et al. 2003), and TRP melastatin 8 (TRPM8) determines whether temperatures are considered cool or cold

(McKemy et al. 2002; Peier et al. 2002; Daniels and McKemy 2007). However, to date, there is no evidence that TRPM8 is involved in the mechanisms of acute OPN.

Menthol, a potent TRPM8 agonist, has long been known to induce or intensify cold sensations by interacting with the peripheral cold receptor, TRPM8 (McKemy et al. 2002; Peier et al. 2002; Knowlton et al. 2010). The tongue is a well-characterized sensory organ, and TRPM8 is present in sensory lingual nerve fibers that mainly project from the trigeminal ganglion where they function as cold and menthol receptors on the tongue (Abe et al. 2005).

On the basis of these observations, we hypothesized that TRPM8 is involved in the mechanisms of acute OPN, especially marked sensitivity to cold. We tested this hypothesis by topically applying varying concentrations of menthol, a TRPM8 agonist, to the patients' tongue before and after oxaliplatin infusions to determine their sensitivity to cold sensation. The minimum concentration of menthol to evoke cold sensation (CS) at the menthol application site was determined as the cold sensation detection threshold (CDT).

The conventional clinical grading system was used to assess the severity of neurotoxicity in relation to CDT. Patients also completed self-report ratings of their sensitivity to cold sensation, and the results of these objective and subjective findings were compared.

Materials and Methods

Subjects and treatment regimen

A total of 76 subjects were enrolled in this study: 40 healthy subjects (24 women, 16 men; median age, 54 years; range, 22–85) and 36 patients (22 women, 14 men; median age, 57 years; range, 33–80) with advanced-stage colorectal cancer who received standard oxaliplatin in combination with infusional 5-fluorouracil/leucovorin (FOLFOX) as a first-line treatment. In the FOLFOX regimens, oxaliplatin (modified FOLFOX 6, 85 mg/m^2) was given intravenously over 2 h on day 1 in conjunction with leucovorin (200 mg/m^2) and followed by a 5-fluorouracil (5-FU) bolus injection (400 mg/m^2), repeated every 2 weeks. A continuous 24-h infusion of 5-FU (600 mg/m^2) was given over days 1 and 2. On day 2, leucovorin (200 mg/m^2; over 2 h) and 5-FU bolus (400 mg/m^2) were given intravenously.

The subjects did not consume any spicy food 1 day prior to testing. They were also asked to refrain from eating, drinking, chewing gum, brushing their teeth, and using mouthwash for 2 h before testing, and we verified that the participants had observed these restrictions at the beginning of each session.

The present study was conducted in accordance with the Declaration of Helsinki for the care for human studies adopted by the Ethics Committee of Higashi-Asahikawa Hospital. All patients provided written informed consent.

Assessment of menthol in experiments 1 and 2

A solution of 5% L-menthol (from dry crystals; MERCK, Tokyo, Japan) was prepared in warm distilled water (41°C) at the time of application, and this solution was further diluted in warm distilled water to yield menthol solutions of 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, and 1% (0.32 mM, 0.64 mM, 3.2 mM, 6.4 mM, 32 mM, and 64 mM, respectively). These solutions were topically applied with a cotton swab to the dorsal anterior tongue in two experiments (Fig. 1a). In experiment 1, the six different menthol solutions were administered to healthy subjects and patients with colon cancer prior to oxaliplatin exposure, and their subjective ratings of cold sensitivity were recorded. In experiment 2, patients were examined for alterations in the menthol-induced cold sensations before and 5–6 h after the patients receiving individual oxaliplatin infusions. The menthol concentrations used in this study were based on a previous human study (Albin et al. 2008). The vehicle control (warmed distilled water) was applied in the same manner.

Both experiments were performed in a room maintained at a constant temperature (22 ± 1°C) and a relative humidity of 55 ± 5%. The menthol testing was performed by two investigators (TK and MS) on all participants. Neither the individuals nor the investigator were aware of whether menthol or the vehicle was applied first because the substances were encoded by a technical assistant.

Cold sensations and Cold sensation detection threshold

The highest and lowest concentrations of the menthol solutions were set at 1% and 0.005%, respectively. Starting at the lowest and increasing to the highest menthol concentration, the solutions were applied with an interstimulus interval of 10 sec. For each stimulus, the subject was instructed to push a button as soon as he or she detected a CS (CDT). The CDT was considered the minimum menthol concentration. When no threshold was obtained, the highest concentration tested (1%) was entered as the threshold value.

Assessment of neurotoxicity

The National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 was used to evaluate the severity of neurotoxicity: grade 1 (mild), loss of deep tendon reflexes or paresthesia not interfering with function; grade 2 (moderate), sensory alteration or paresthesia interfering with function but not activities of daily living; grade 3 (severe), sensory alteration or paresthesia interfering with activities of daily living; and grade 4, disabling (Trotti et al. 2003).

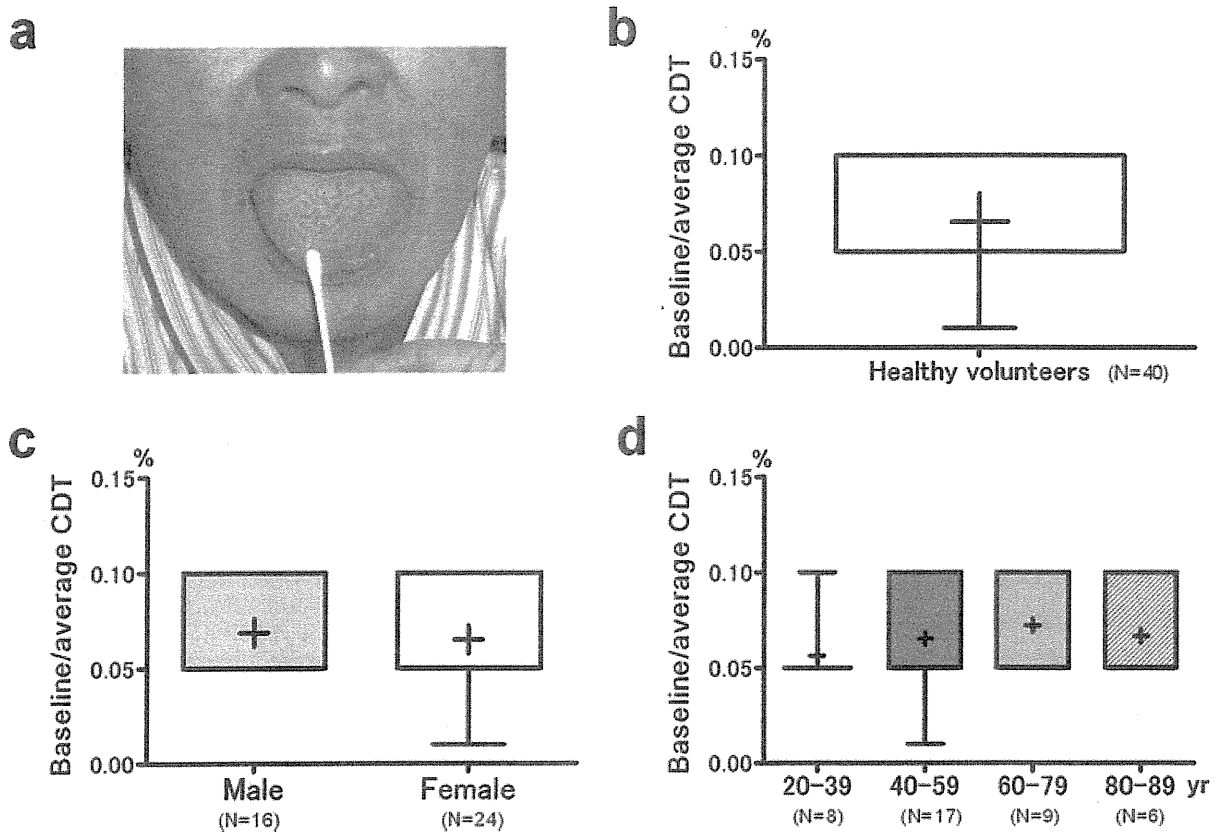

Figure 1. Effects of menthol on cold sensation and the detection threshold in healthy human subjects. (a) The menthol solution was topically applied with a cotton swab to the dorsal anterior tongue. (b) The mean baseline cold sensation detection thresholds (CDTs) in healthy human volunteers (n = 40) were 0.01% (1 of 40 subjects), 0.05% (26 of 40), and 0.1% (13 of 40). The overall mean CDT was 0.068 ± 0.026% (mean ± SD). (c) Significant sex difference in mean baseline CDT was not found. (d) Significant age difference in mean baseline CDT was not found. Cross is the mean of CDT.

Statistics

The effects of oxaliplatin were analyzed by the nonparametric Wilcoxon t-test for paired samples. In all of the statistical analyses, significance was determined using an alpha level of 0.05. All statistical procedures were performed using the IBM-SPSS software package version 18.0J for Windows (Tokyo, Japan) and the GraphPad Prism 4 statistics program (GraphPad Software, Inc., San Diego, CA).

Results

Effects of menthol on CS and CDT in healthy human subjects and patients with colon cancer (experiment 1)

All subjects noticed a significant feeling of coldness at the menthol application site. The CS occurred within the first 3 sec, reached an intensity plateau at approximately 5 sec and then disappeared within 10 sec. The intensity of the CS increased in a dose-dependent manner. None of the subjects experienced a CS when the vehicle control was applied. The mean baseline CDTs in healthy human volunteers were 0.01% (1 of 40 subjects), 0.05% (26 of 40), and 0.1% (13 of 40). The mean CDT was 0.068 ± 0.026% (SD) (Fig. 1b). To assess reproducibility, 40 healthy subjects were retested, and their CDTs were found not to differ significantly from the previous testing. Significant sex and age differences in mean baseline CDTs were not found as well (Fig. 1c and d). No serious adverse events occurred during the study and all doses of menthol were well tolerated.

The mean CDT in patients with colon cancer who had never received any chemotherapy was 0.067 ± 0.025% (n = 12). No significant difference in mean baseline CDT was observed between healthy subjects and patients with colon cancer. In addition, no serious adverse events occurred during the study and all doses of menthol were well tolerated.

Changes in the CDT before and after oxaliplatin administration (experiment 2)

Figure 2a shows the CDTs that were obtained before and after the first oxaliplatin administration in patients who had

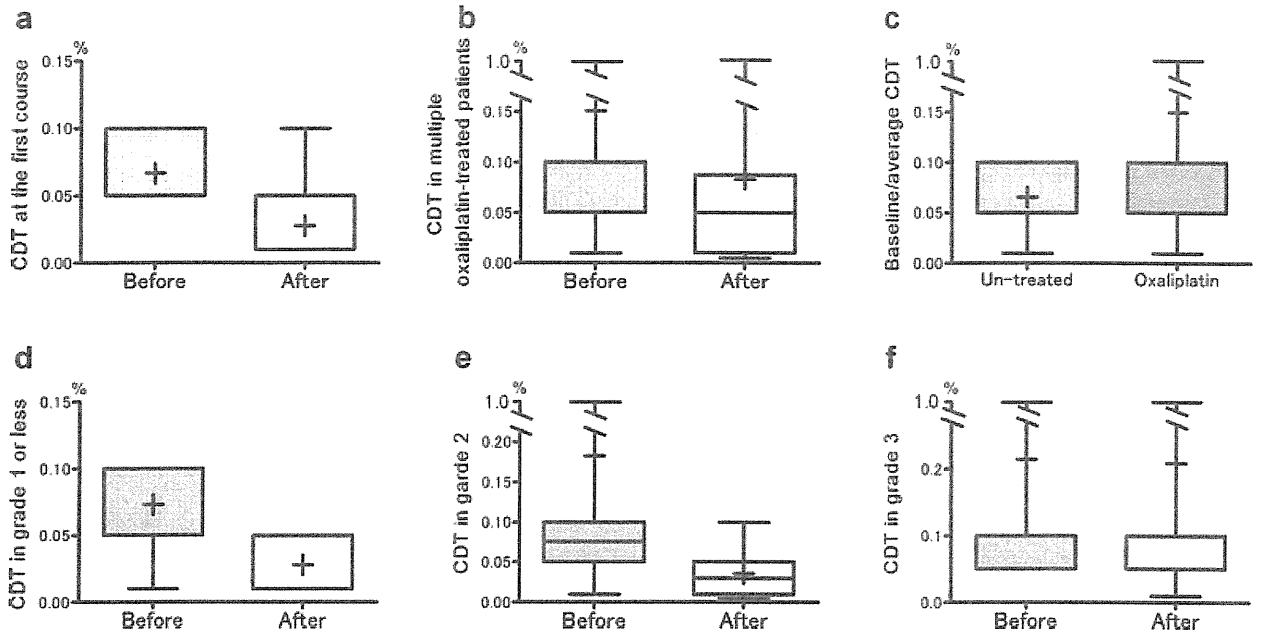

Figure 2. Changes in the cold sensation detection threshold (CDT) before and after oxaliplatin administration. (a) The CDT was determined by applying menthol before and after the first oxaliplatin administration. The CDT significantly decreased from 0.067 ± 0.025% (mean ± SD) to 0.028 ± 0.029% ($n = 12$, $P = 0.0025$). (b) Changes in the CDT before and after oxaliplatin administration in patients previously treated with oxaliplatin. The CDT significantly decreased from 0.151 ± 0.263% to 0.083 ± 0.198% ($n = 24$, $P = 0.0004$). (c) The mean baseline CDT was significantly higher in patients previously treated with oxaliplatin ($n = 24$) than in untreated subjects ($n = 52$) (0.151% vs. 0.066%, $P = 0.0225$). (d) The CDT was measured before and after oxaliplatin was administered to patients who had grade 1 or less neurotoxicity. The CDT significantly decreased from 0.073 ± 0.034% to 0.028 ± 0.021% ($n = 9$, $P = 0.0126$). (e) The CDT was measured before and after oxaliplatin was administered to patients who had grade 2 neurotoxicity. There was no significant difference in the CDTs ($n = 8$; before, 0.183 ± 0.332%; after, 0.036 ± 0.033%; $P = 0.022$). (f) The CDT was obtained before and after oxaliplatin was administered to patients who had grade 3 neurotoxicity. There was no significant difference in the CDTs ($n = 7$; before, 0.214 ± 0.347%; after, 0.209 ± 0.351%; $P = 1.0$). Cross is the mean of CDT.

never received chemotherapy. All but one patient were hypersensitive to menthol as indicated by a significant decrease in the CDT from 0.067 ± 0.025% to 0.028 ± 0.029% ($n = 12$, $P = 0.0025$). The CDTs were also measured before and after oxaliplatin administration in patients who had previously received oxaliplatin ($n = 24$, median, 330 mg/m^2; range, 85 – 2450 mg/m^2). Under these conditions, the CDT significantly decreased from 0.151 ± 0.263% to 0.083 ± 0.198% ($n = 24$, $P = 0.0004$) (Fig. 2b). Taken together, these findings show that the mean baseline CDT was significantly higher in patients previously treated with oxaliplatin ($n = 24$) than in untreated subjects ($n = 52$) (0.151% vs. 0.066%, $P = 0.0225$).

When the relationship between the CDTs and the CTCAE neurotoxicity ratings in oxaliplatin-treated patients was evaluated, the CDTs were found to be significantly decreased in patients who had grade 1 or less neurotoxicity (from 0.073% to 0.028%) ($n = 9$, $P = 0.0126$) (Fig. 2d), and grade 2 (from 0.183% to 0.036%) ($n = 8$, $P = 0.022$) (Fig. 2e), but not in those with grade 3 neurotoxicity (from 0.214% to 0.209%) ($n = 7$, $P = 1.0$) (Fig. 2f).

Discussion

Our results indicate a potential correlation between TRPM8 activity and OPN, especially in acute hypersensitivity to CS, and that acute changes in CDT may facilitate the identification of early OPN. In chemotherapy-naïve patients, significant sensitivity to topical menthol developed after the first oxaliplatin infusion, suggesting that oxaliplatin had indeed induced cold hypersensitivity. In contrast, patients with previous oxaliplatin exposure showed reduced cold hypersensitivity. With regard to the relationship between the CDT and neurotoxicity grade, we found that mild or moderate neurotoxicity was associated with significant changes in the CDT, while severe neurotoxicity was not associated with marked changes in the CDT. Whether the CDT remains unaltered in oxaliplatin-treated patients who do not develop OPN despite chronic oxaliplatin exposure requires further investigation. Nonetheless, these findings suggest that the CDT is a sensitive marker of early oxaliplatin-induced sensory disturbances.

Menthol activates the cold-transducing Ca^{2+} ion channel TRPM8 and increases cold-evoked currents (McKemy et al.

2002; Peier et al. 2002), and TRPM8 is naturally expressed sensory neurons (Reid et al. 2002; Abe et al. 2005; Kobayashi et al. 2005; Madrid et al. 2006). These TRPM8-expressing sensory neurons project into the superficial laminae of the spinal cord dorsal horn (Dhaka et al. 2008; Wrigley et al. 2009) that contains cold-sensitive neurons that project into the spinothalamic tract (Craig and Dostrovsky 2001). Thus, the cold-induced paresthesias after oxaliplatin administration that were accentuated by menthol might be mediated via the activation of TRPM8-expressing innocuous cold receptors, assuming that the receptors access central neurons. Although the precise mechanisms underpinning OPN are still uncertain, this study may serve as an entry point in furthering the mechanistic understanding of OPN. Oxaliplatin has also been shown to modify intracellular Ca^{2+} handling within the cell bodies of cultured neurons (Grolleau et al. 2001). A more recent study cited a possible mechanism for some of the oxaliplatin-induced effects that is related to the modification of surface charges around the ion channel: either due to extracellular Ca^{2+} chelation or binding of a charged biotransformation product of oxaliplatin to the channel (Broomand et al. 2009). In addition, the prospective CONcePT study confirmed that OPN could be strongly attenuated by pre- and post-treating patients with Ca^{2+} and Mg^{2+} infusions (Gamelin et al. 2008). These findings suggest a mode of action that involves a Ca^{2+}-dependent mechanism in OPN. Therefore, the Ca^{2+} ion channel TRPM8 appears to be a good candidate for understanding the Ca^{2+}-dependent mechanism in OPN.

The TRP ion channel family consists of approximately 28 mammalian cation channels (Gaudet, 2008; Talavera et al. 2008; Eid and Cortright, 2009) that are involved in a wide range of physiological and pathophysiological processes including taste, thermosensation, pain, and cell cycle regulation. The TRP ion channels present a novel mechanism for controlling Ca^{2+} transients in human neurons and represent potential targets for regulating neurite proliferation and outgrowth. Recent studies have shown that regulating TRPM8 ion channels may be a way of controlling Ca^{2+} transients in human neurons. We, therefore, hypothesized that oxaliplatin could alter calcium-sensitive voltage-gated Na channels through a pathway that involves Ca^{2+} ions that are likely mobilized by TRPM8.

Several limitations should be considered in light of our results. Firstly, we did not conduct additional follow-up of CDT after oxaliplatin infusion. Such data would provide a context for the length of time it takes for the CDTs to return to normal and would be very useful from a clinical translation standpoint to approximate the outcome of patients after oxaliplatin infusion. This approach will be incorporated into our next protocol. Second, we compared our CDT findings against the CTCAE grading system that is a gross general measure of neuropathy impairment (Trotti et al. 2003) that precludes specific measurement of cold allodynia symptoms. Hence, our menthol testing needs validation against a testing method that provides an objective evaluation of cold allodynia/parasthesia, preferably the gold standard of CS, such as quantitative sensory testing. The validation of the menthol testing using quantitative sensory tests will be one of the important future studies. In addition, although our healthy subjects and chemotherapy-naïve patients were similar in age, sex, and baseline CDTs, having colon cancer patients as controls rather than healthy volunteers would have established equivalency at baseline by accounting for the potential influence of cancer-specific changes on CDTs. Future studies would benefit from conducting additional evaluations of CDTs after oxaliplatin infusion, performing quantitative sensory testing, and using patients with colon cancer without OPN as controls.

The present data show that menthol may be used to determine and evaluate the neurotoxicity severity score, although the methodology using menthol has not been firmly established. Interestingly, patients with prior oxaliplatin exposure had significantly elevated CDT at baseline, and patients with grade 3 neurotoxicity did not show significant changes in the CDT before and after oxaliplatin administration. These findings suggest that TRM8 may be associated with the chronic stage of OPN. Unfortunately, in this study, these patients were not prospectively monitored for changes in the CDT during and after a long period of oxaliplatin treatment therefore, we could not confirm whether or not the CDT increased with OPN progression. A prospective, multicenter, randomized, double-blind study is needed to investigate the possibility of CDT as a diagnostic marker for OPN.

In conclusion, our findings indicate that OPN may be associated with TRPM8 in acute hypersensitivity to CS, and that additional studies on TRPM8 will enhance our understanding of the mechanisms of OPN. Further, our study demonstrates the feasibility of undertaking CDT test in a clinical setting to facilitate the identification of early neurotoxicity, although larger trials need to be conducted to confirm our findings.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Daikenchuto (TU-100) ameliorates colon microvascular dysfunction via endogenous adrenomedullin in Crohn's disease rat model

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Abstract
Background Daikenchuto (TU-100), a traditional Japanese medicine, has been reported to up-regulate the adrenomedullin (ADM)/calcitonin gene-related peptide (CGRP) system, which is involved in intestinal vasodilatation. The microvascular dysfunction of the intestine in Crohn's disease (CD), due to down-regulation of the ADM/CGRP system, is etiologically related to the recurrence of CD. Therefore, we investigated the vasodilatory effect of TU-100 in a CD rat model.
Methods Colitis was induced by the rectal instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in rats. Laser Doppler blood flowmetry was used to measure colonic blood flow. ADM, CGRP, and their receptors in the ischemic colon were measured by reverse transcription polymerase chain reaction (RT-PCR) and enzyme immunoassays. Additionally, we determined whether the intestinal epithelial cell line IEC-6 released ADM in response to TU-100.
Results TU-100 increased blood flow in ischemic segments of the colon but not in hyperemic segments. Pretreatment with an antibody to ADM abolished the vasodilatory effect of TU-100. CGRP levels and βCGRP mRNA expression were decreased in the ischemic colon, while protein and mRNA levels of ADM were unchanged. Hydroxy α-sanshool, the main constituent of TU-100, was the most active component in improving blood flow. Additionally, both TU-100 and hydroxy α-sanshool enhanced the release of ADM from IEC-6 cells.
Conclusions In the ischemic colon, endogenous βCGRP, but not ADM, was decreased. Thus, it was concluded that TU-100 ameliorated microvascular dysfunction by the up-regulation of endogenous ADM in the CD rat model. TU-100 may be a possible therapeutic agent for gastrointestinal ischemia-related diseases including CD.

Keywords Daikenchuto (TU-100) · Adrenomedullin · Crohn's disease · Ischemia · Colonic blood flow

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Introduction

Postoperative recurrences of Crohn's disease (CD) at the anastomotic site are common, often requiring further abdominal surgery for anastomotic strictures. Indeed, within 1 week after operation, histopathological findings of recurrent CD may be seen [1], and the incidence of endoscopically detected recurrence reaches 70–90% at 1 year postoperatively [2, 3], thereby predisposing such patients to develop symptomatic stenosis. It has been reported that blood flow is decreased by more than 50% in the terminal ileum and colon of CD patients [4, 5]. Decreased blood flow is one of the factors involved in recurrence at the anastomotic site [6]. A potent endogenous

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microvascular dilator, calcitonin gene-related peptide (CGRP), a neuropeptide produced by the nervous system of the gut, is decreased in animal models of CD and in CD patients [7]. Moreover, it has been reported that ischemia plays a key role in the pathogenesis of CD. In fact, several clinicians and researchers who have investigated the vascular contribution to the pathogenesis of CD have suggested the possibility of vasodilators as therapeutic agents [8, 9].

Daikenchuto (TU-100), a traditional Japanese medicine (such medicines are known as Kampo), is manufactured as a recognized prescription drug with standardized quality and quantities of ingredients [10]. In Japan, TU-100 is prescribed to improve gastrointestinal motility and prevent postoperative adhesion and paralytic ileus after abdominal surgery, and its clinical efficacy has been defined [11–15]. Moreover, TU-100 had a significant promotility effect in the small bowel and in ascending colon transit in healthy subjects compared with placebo in a randomized, double-blind study in the United States [16]. Recently, we reported that TU-100 exerted therapeutic effects in a CD mouse model via the enhancement of endogenous adrenomedullin (ADM) in the intestine [17]. TU-100 directly stimulated intestinal epithelial cells, resulting in increased ADM production. We also reported that the administration of TU-100 to normal rats increased small and large intestinal blood flow via CGRP systems [18, 19].

ADM, like CGRP, is a peptide of the calcitonin family and a potent endogenous vasodilator [20, 21]. ADM is ubiquitous in the intestinal tract and plays physiologically important roles in the microcirculation of the intestine [22, 23]. Therefore, up-regulation of endogenous ADM may attenuate the microvascular dysfunction in the CD intestine.

CGRP has two different isoforms, αCGRP and βCGRP. The former is mainly expressed in the sensory afferent neurons of the extrinsic nervous system and the latter in the intrinsic primary afferent neurons [24–26]. A decrease in CGRP has been reported in CD and this decrease might contribute to microvascular dysfunction in the CD intestine [7, 27]. However, there is no information on which types of CGRP are decreased in the CD intestine.

It is our hypothesis that TU-100 enhances endogenous ADM, which increases intestinal blood flow to compensate for the decrease due to decreased CGRP in the CD intestine. In this study, we examined the vasodilatory effects of TU-100 in a rat model of CD produced using 2,4,6-trinitrobenzenesulfonic acid (TNBS), identified the active constituents of TU-100, and explored potential mechanisms related to the ADM/CGRP system, using antibodies and an intestinal epithelial cell line. Moreover, we investigated which isoform of CGRP was decreased in the CD intestine.

Materials and methods

Animals

Male Sprague–Dawley rats (Japan SLC, Shizuoka, Japan) weighing 300–400 g were used. The animals were allowed free access to water and standard laboratory food and were housed at a temperature of 23 ± 2°C, relative humidity of 55 ± 10%, and a 12-h light: 12-h dark cycle with lights on from 0700 to 1900 hours daily. All experimental procedures were performed according to the "Guidelines for the care and use of laboratory animals" of Asahikawa Medical University and Tsumura Research Laboratories. All animal procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Reagents

TU-100, obtained from Tsumura (Tokyo, Japan), is manufactured as a water-soluble extract (containing processed ginger, ginseng radix, Japanese pepper, and maltose syrup [88.9%]) under strictly controlled conditions. The qualities of these raw materials were tested according to the standards of the Japanese Pharmacopoeia and Tsumura, and a routine chemical analysis was performed (Fig. 1). Maltose and 6-shogaol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hydroxy α-sanshool (HAS) was extracted from Japanese pepper at Tsumura. Ginsenoside Rb1 was purchased from Extrasynthese (Genay, France), rabbit anti-ADM polyclonal IgG was purchased from Peninsula Laboratories (Belmont, CA, USA), and rabbit IgG was purchased from Abcam (Cambridge, UK) as an isotype-matched control.

Colonic blood flow

Total transmural colonic blood flow was measured using a laser Doppler flowmeter (ALF21N; Advance, Tokyo, Japan) as described previously [19]. Briefly, rats were anesthetized with urethane (900 mg/kg i.v.), α-chloralose (45 mg/kg i.v.), and butorphanol (Bristol-Myers Squibb, New York, NY, USA; 1 mg/kg i.m.). A tracheotomy was performed, and rats were ventilated artificially (respiratory rate 60 breaths/min) and placed on a heating pad. The left cervical artery was cannulated and connected to a transducer (P23XL; Nihon Kohden, Tokyo, Japan) to monitor systemic arterial blood pressure (AP) and heart rate (HR). An oral thermometer was placed in the mouth and body temperature was maintained at 37 ± 0.5°C. After exposing the colon by a midline laparotomy, a cannula (18G) was inserted via the cecum into the proximal colon to facilitate injection of the test substances. The distal colon was exposed and covered with plastic wrap to prevent the tissue

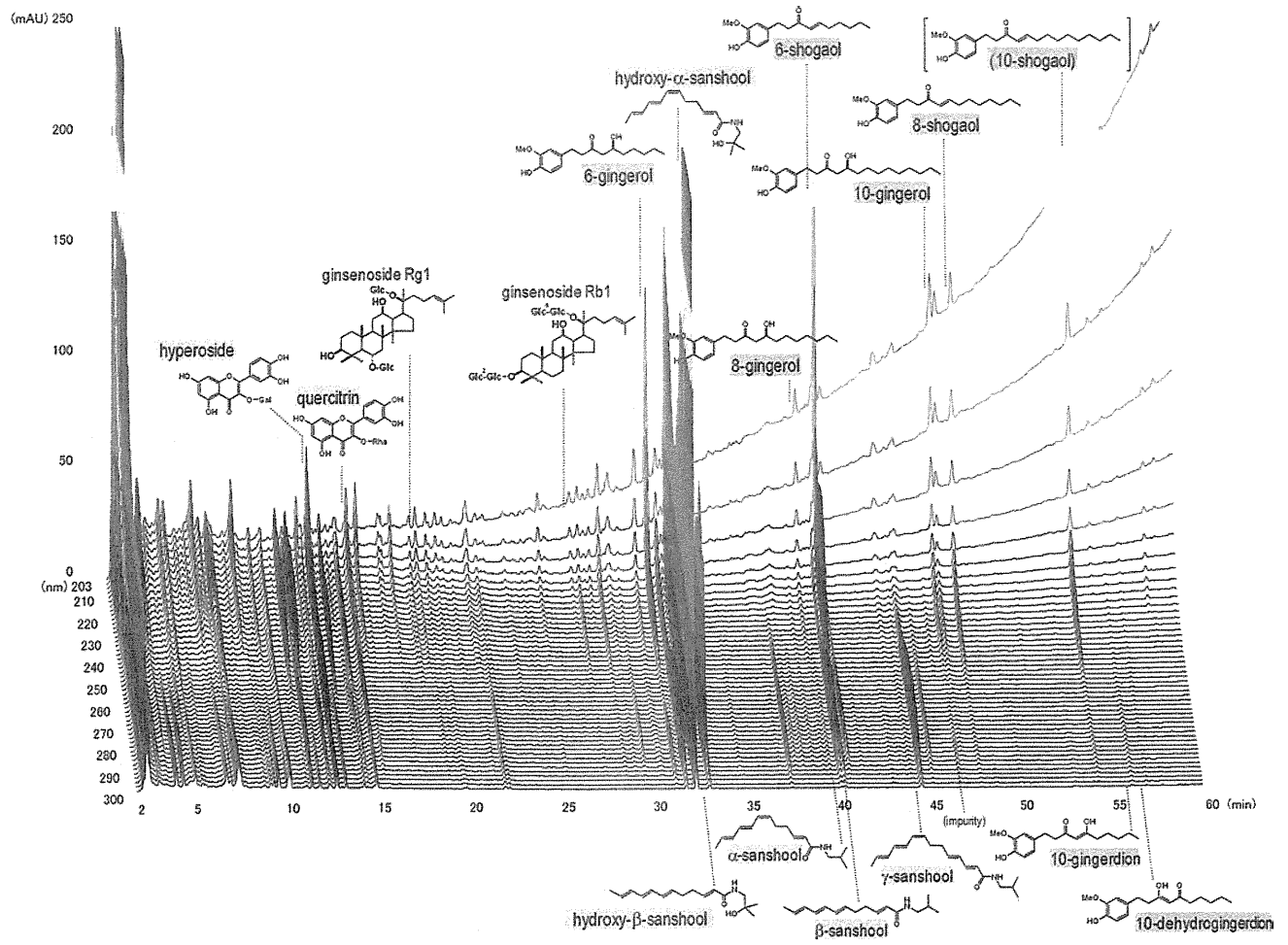

Fig. 1 Three-dimensional high-performance liquid chromatograph of TU-100

from becoming dehydrated. A fiberoptic probe was positioned 4 mm above the surface of the distal colon. Blood flow, AP, and HR were monitored continuously by a PowerLab data-sampling unit (PowerLab 8/30; AD Instruments, Tokyo, Japan) and recorded. Colonic vascular conductance (CVC) was measured as an index of total transmural colonic blood flow [28]. CVC was calculated as the quotient of mean blood flow divided by mean AP.

Effects of antibody and constituents of TU-100 on CVC

TU-100 (900 mg/5 mL/kg) or distilled water was injected into the lumen of the colon after confirming a stable blood flow. The ADM antibody (50 μg/kg) was injected through an intravenous polyethylene tube in the right jugular vein. Fifteen minutes later, TU-100 (900 mg/5 mL/kg) was injected. In another set of experiments, the effects of specific constituents of TU-100 on CVC were examined. The CVC responses to HAS, ginsenoside Rb1, 6-shogaol, and maltose were examined using doses of 0.3, 1, 0.2, and

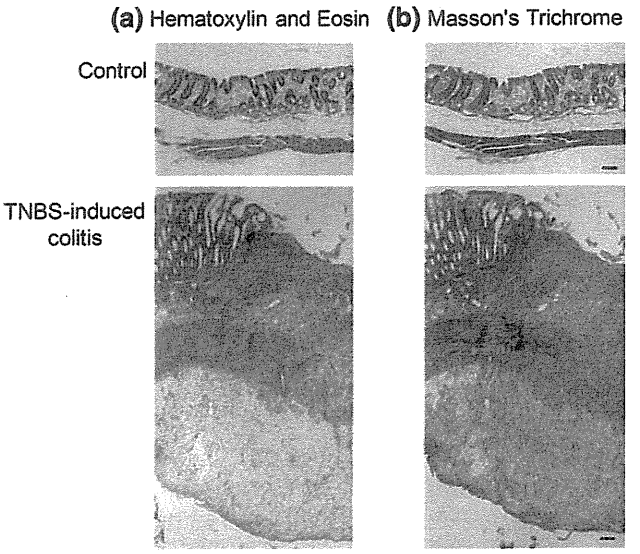

Fig. 2 Histology of segments of ischemic colon in rats with colitis. Sections of the colon 10 days after instillation of 2,4,6-trinitrobenzenesulfonic acid (*TNBS*) or 0.9% NaCl (control) were stained with hematoxylin and eosin (**a**) or Masson's trichrome (**b**). *Scale bar* indicates 100 μm

800 mg/kg, respectively. The test substances were dissolved in 1% Tween 80 and administered intraluminally. The blood flow and AP were measured at 15-min intervals after intracolonic administration.

ADM production assay

ADM enhancing activity was assessed as described previously. Namely, the rat intestinal epithelial cell line IEC-6 was obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mmol/L hydroxyethylpiperazine ethanesulfonic acid (HEPES). IEC-6 cells between the 30th and 37th passages were plated in 96-well flat-bottom microtiter plates at 1×10^4 cells/well in DMEM supplemented with the same additives, allowed to settle overnight, and then culture fluids were replaced with fresh DMEM containing 3% FBS and the test sample. Extract samples were suspended in dimethyl sulfoxide (DMSO) at 100 mg/mL, diluted in DMEM, passed through a 0.45-μm filter, and then added to the cultures. Cells were incubated for an additional 24 h, and ADM in the supernatants was assessed by using an enzyme immunoassay (EIA) kit specific for rat ADM according to the procedure provided by the manufacturer (Phoenix Pharmaceuticals, Burlingame, CA, USA). The lowest level of detection was 10 pg/mL.

Induction of colitis

Colitis was induced by a procedure described previously, with a slight modification [29]. In brief, with the rat under ether anesthesia, 25 mg TNBS (Tokyo Chemical Industries, Tokyo, Japan) in 0.5 mL of 50% ethanol was instilled transanally into the lumen of the distal colon using a flexible feeding tube (RZ-2; CLEA Japan, Suita, Japan). All experiments were performed 10 days after TNBS or 0.9% NaCl (control) administration. The colonic segments that exhibited less than 0.06 CVC showed prominent fibrous thickening histologically, without exception. We selected these segments as ischemic colons, and studied them histologically and biochemically as described below.

Histologic examination

The colonic tissues from control rats and TNBS-induced colitis rats were excised, opened by a longitudinal incision, rinsed with 0.9% NaCl, and weighed. Representative colonic segments were fixed in 4% buffered paraformaldehyde and embedded in paraffin. The histology was evaluated using hematoxylin and eosin and Masson's trichrome stains.

Immunohistochemistry for ADM

Small and large intestinal tissues obtained from normal rats were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and cut into 5-μm sections. Subsequently, serial sections were stained with rabbit polyclonal IgG antibody against ADM (Peninsula Laboratories) as the primary antibody, and peroxidase-conjugated goat anti-rabbit IgG (DAKO, Glostrup, Denmark) was used as a secondary antibody. The reaction was developed by adding 3,3′ diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) solution. All incubations were done for 20 min, with saturated antibody concentrations and followed by two washes.

Immunoreactive CGRP and ADM in colon

Isolated segments of colon were homogenized in 2 N acetic acid at a concentration of 10 mg/mL, heated for 15 min at 90°C, and centrifuged at $10{,}000 \times g$ for 20 min at 4°C. The supernatant was applied to an activated C18 Sep column (Phoenix Pharmaceuticals) and eluted with 60% acetonitrile in 1% trifluoroacetic acid. The eluate was vacuum-dried and stored at −80°C until assay. The CGRP level was measured using a rat CGRP EIA Kit (Peninsula Laboratories). The lowest level of detectability for CGRP was 2 pg/mL. The intraassay coefficient of variation was 2.7%, and the interassay coefficient of variation was 9.3%. Cross-reactivity between rat αCGRP and βCGRP was 100%. ADM was quantified by using the EIA kit as described above.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Segments of normal or ischemic colon were homogenized in QIAzol Lysis Reagent (Qiagen, Valencia, CA, USA), and total RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Messenger RNA expressions of αCGRP, βCGRP, ADM, ADM2, calcitonin receptor-like receptor (CRLR), receptor activity-modifying protein (RAMP)1, RAMP2, and RAMP3 were measured by real-time quantitative RT-PCR (TaqMan gene expression assays) and an ABI Prism 7900 sequence detection system (Applied Biosystems, Warrington, UK). Sample-to-sample variation in RNA loading was controlled by comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative quantitation of gene expression was performed using the ΔΔ CT (change in the cycle threshold) method.

Statistical analysis

All values are expressed as means ± SEM. Statistical significance was evaluated by two-way analysis of variance

(ANOVA) followed by Dunnett's test or Student's *t*-test. For all tests, significance was accepted at $p < 0.05$.

Results

Histology of ischemic colon

The disease states of our model were confirmed by histological observation. Figure 2 shows the histology of representative segments of the ischemic colon on day 10 after instillation of TNBS or saline (control). In rats with TNBS-induced colitis, transmural inflammation was present in all layers of the bowel wall, with a marked increase in the thickness of the mucosal and muscular layers. The affected colon wall consisted of granulomatous tissue in which fibroblasts and fibrosis were present in the submucosa together with regenerative changes in the overlying epithelium. Masson's trichrome stain showed extensive fibrosis in the ischemic colon.

TU-100 had vasodilatory effect on the ischemic segment of TNBS-induced colitis, suggesting the possible involvement of endogenous ADM.

Colonic blood flow after intraluminal administration of TU-100 (900 mg/5 mL/kg) to TNBS-induced colitis rats is shown in Fig. 3a. The involved segments of the ischemic colon (basal CVC $\leq$ 0.06) and the hyperemic colon (basal CVC $\geq$ 0.20) in colitis rats were identified by measurement of blood flow; in comparison, that of normal rats was 0.10 $\pm$ 0.003 (basal CVC). Colonic blood flow in the ischemic site was increased to the normal CVC range within 15 min after administration of TU-100, and the effect lasted throughout the data acquisition period. By contrast, blood flow in the hyperemic site was not changed by TU-100 administration. Mean AP and HR were unchanged after administration of TU-100 (data not shown). As shown in Fig. 3b, the TU-100-induced vasodilatation in the ischemic segments was substantially abolished by pretreatment with the antibody against ADM, except for the initial increase in blood flow for 15–30 min after TU-100 administration. Figure 4 shows the vascularity of an ischemic colon segment before and after TU-100 treatment. Microvascular blood flow was evidently increased by TU-100 administration.

CGRP and ADM in ischemic colon

As shown in Fig. 5, the CGRP level in TNBS-induced colitis rats was lower than that in control rats (23.9 $\pm$ 6.7 vs. 80.2 $\pm$ 12.4 ng/g tissue, $p < 0.05$). In contrast, the ADM level in colitis rats was unchanged compared with that in control rats (1.2 $\pm$ 0.1 ng/g tissue).

Expressions of CGRP and ADM mRNA in the colon are shown in Table 1. Judging from the difference in cycle threshold values, the mRNA expression of βCGRP was up to 23 times greater than that of αCGRP in the colons of control rats. The level of βCGRP mRNA in the colons of colitis rats was lower than that in control rats (0.62 $\pm$ 0.12 vs. 1.00 $\pm$ 0.09, $p < 0.05$). In contrast, the level of αCGRP mRNA was not significantly decreased after treatment with TNBS. The level of ADM2 mRNA in the colitis rats was greater than that in control rats (4.72 $\pm$ 1.36 vs.

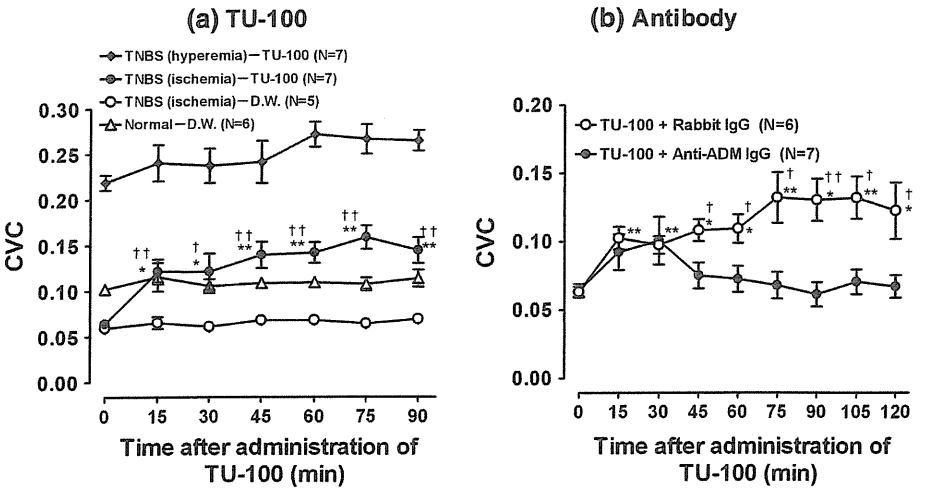

Fig. 3 Involvement of endogenous adrenomedullin (*ADM*) in vasodilatory effect of TU-100 in colitis rats. **a** TU-100 and vehicle (distilled water [*D.W.*]) were evaluated. $N = 5$–7. **b** Rabbit polyclonal IgG specific to ADM was infused intravenously at 50 μg/mL/kg 15 min before TU-100 administration. Rabbit non-specific IgG was injected as the matched control ($N = 6$–7 rats). Factorial two-way analysis of variance (ANOVA) revealed significant effects of group [$F(2, 125) = 23.70$ $p < 0.0001$] and time [$F(5, 125) = 6.08$, $p < 0.0001$]. $^{*}p < 0.05$, $^{**}p < 0.01$ versus pre-administration (0 min) (Dunnett's test). $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus no-antibody control, respectively (Student's *t*-test). *CVC* Colonic vascular conductance

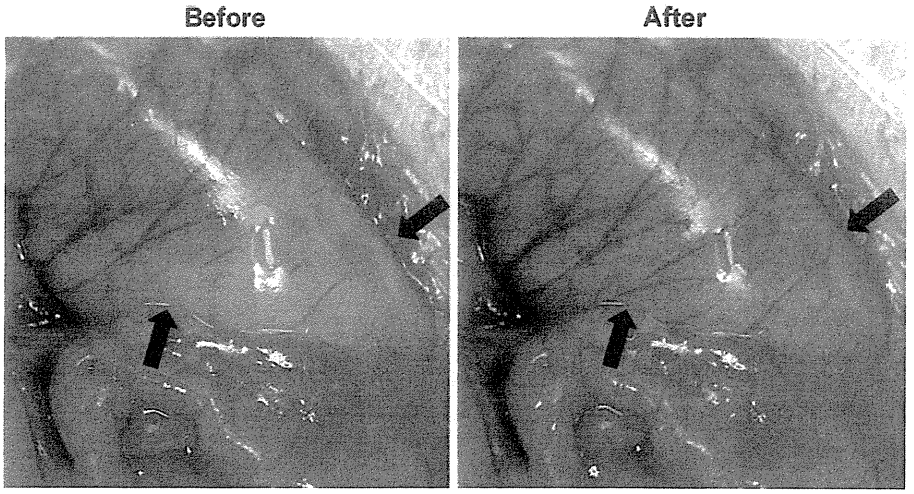

Fig. 4 Vasodilatation by TU-100 in TNBS-treated colon. Photographs show the colon exposed by a midline laparotomy before and 15 min after administration of TU-100. Vasodilated microvessels are visible in the ischemic site of a TNBS-treated rat (*arrows*)

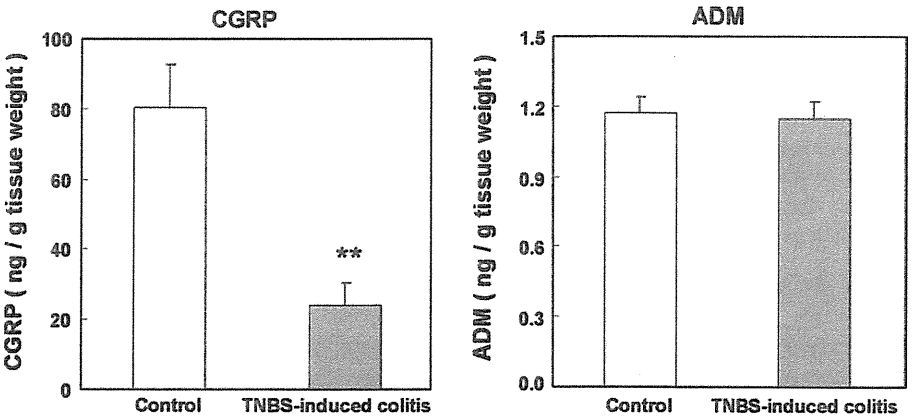

Fig. 5 Down-regulation of calcitonin gene-related peptide (*CGRP*), but not ADM, concentration in segments of ischemic colon. Ten days after colonic instillation of TNBS or 0.9% NaCl (control), colon segments were isolated. CGRP and ADM in each sample were measured using an enzyme immunoassay (EIA) kit. $N = 6$. $^{**}p < 0.01$ versus control (Student's *t*-test)

Table 1 mRNA expressions in segments of ischemic colon

	ΔΔ CT		Relative mRNA expression	
	Control	TNBS-induced colitis	Control	TNBS-induced colitis
αCGRP	14.34 ± 0.25	13.12 ± 0.59	1.00 ± 0.16	3.05 ± 1.09
βCGRP	9.54 ± 0.14	10.40 ± 0.39*	1.00 ± 0.09	0.62 ± 0.12*
ADM	6.00 ± 0.12	5.60 ± 0.27	1.00 ± 0.09	1.39 ± 0.28
ADM2	12.66 ± 0.33	10.75 ± 0.41*	1.00 ± 0.19	4.72 ± 1.36*
CRLR	7.12 ± 0.13	6.67 ± 0.14*	1.00 ± 0.08	1.35 ± 0.12*
RAMP1	3.82 ± 0.09	3.69 ± 0.23	1.00 ± 0.06	1.14 ± 0.14
RAMP2	6.71 ± 0.06	5.91 ± 0.11**	1.00 ± 0.04	1.70 ± 0.11**
RAMP3	8.22 ± 0.11	6.63 ± 0.33**	1.00 ± 0.04	3.18 ± 0.65**

Quantitative gene expression was analyzed 10 days after administration of TNBS or saline (control) by real-time reverse transcription polymerase chain reaction (RT-PCR) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. The mRNA expressions of αCGRP, βCGRP, ADM, ADM2, CRLR, RAMP1, RAMP2, and RAMP3 were calculated by the change in the cycle threshold (ΔΔ CT) method. Each value is expressed as the mean ± SEM. $N = 6$

CGRP calcitonin gene-related peptide, *TNBS* 2,4,6-trinitrobenzenesulfonic acid, *ADM* adrenomedullin, *CRLR* calcitonin receptor-like receptor, *RAMP* receptor activity-modifying protein

* $p < 0.05$, ** $p < 0.01$ versus control, respectively (Student's *t*-test)

1.00 ± 0.19, $p < 0.05$), while the mRNA expression of ADM was unchanged. Moreover, the mRNA expressions of CRLR, RAMP2, and RAMP3 were significantly increased in the colitis rats. TU-100 treatment had no effect on the mRNA levels of these CGRP receptors.

Immunohistochemistry of ADM in intestinal epithelial cells

To clarify the source of ADM, immunohistochemistry of the small and large intestines isolated from normal rats was investigated. ADM immunoreactivity was observed in the mucosal epithelium and crypts of the intestinal tract (Fig. 6).

ADM enhancement by TU-100 and its components in vitro

TU-100 significantly enhanced ADM release from IEC-6 cells dose-dependently (Fig. 7a). The concentration of ADM in the culture fluid from IEC-6 cells stimulated with 900 μg/mL of TU-100 was 167 ± 6 pg/mL, which was 2.3 times that of the control. Moreover, as shown in Fig. 7b, extracts of processed ginger and Japanese pepper, but not of ginseng radix and maltose syrup, enhanced ADM release. In particular, the ADM concentration after the administration of 100 μg/mL of Japanese pepper extract was 2.4 times that of the control. As indicated in Fig. 7c, HAS significantly enhanced ADM release, dose-dependently, to 2.7 times that of the control at 30 μmol/L.

Effects of constituents of TU-100 on CVC

To identify the active constituents of TU-100, tests were performed using the main components, 6-shogaol (processed ginger), ginsenoside Rb1 (ginseng radix), HAS (Japanese pepper), and maltose (maltose syrup). The CVC was not significantly changed in colitis rats after the intracolonic administration of ginsenoside Rb1, 6-shogaol, maltose, or the vehicle (Fig. 8). In contrast, HAS (0.3 mg/5 mL/kg) increased the CVC at 60, 75, and 90 min compared with the basal value.

Discussion

Blood flow is an important factor in the pathogenesis of CD. Ileal ulcers tend to occur along the mesenteric margin of the bowel wall in CD and in experimental models of inflammatory bowel disease [30–32]. Wakefield et al. [33] proposed the hypothesis that the primary pathological abnormality in CD is in the mesenteric blood supply. They also demonstrated that granulomatous vasculitis caused ischemia in areas of small bowel mucosa supplied by small feeding arteries along the mesenteric margin [34]. Although the overall blood flow was measured, rather than that at only the mesenteric margin, these lines of evidence suggest that the administration of TU-100 may have beneficial effects on an anastomotic site as well as on ulcer healing by improving intestinal blood flow.

The present study demonstrated that the endogenous CGRP level in the colon was significantly lower in

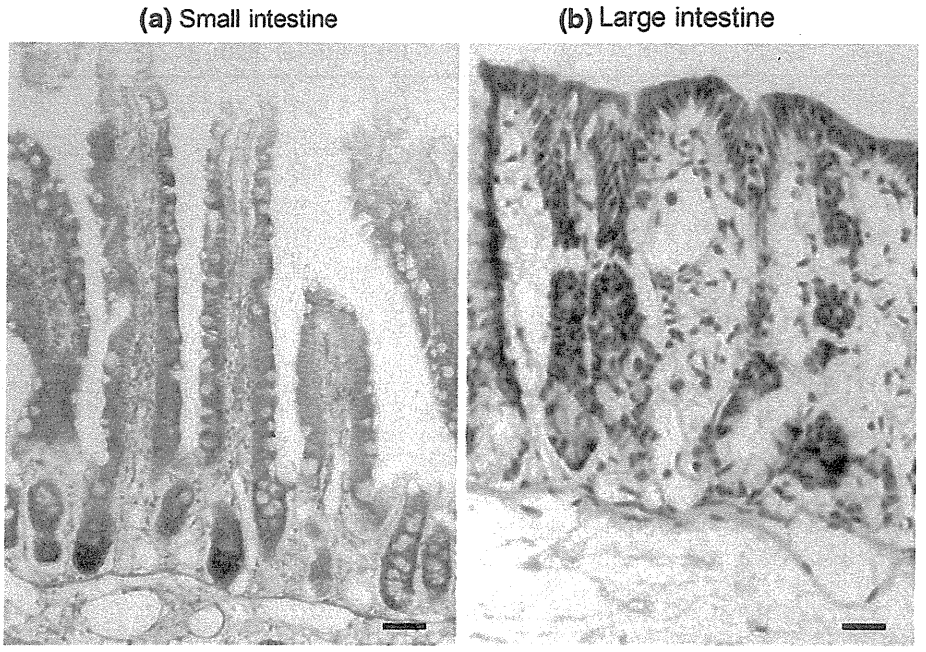

Fig. 6 Immunohistochemistry of ADM in rat intestines. Small and large intestines were obtained from normal rats, and stained with rabbit anti-ADM antibody. *Scale bar* shown in **a** is 50 μm; *scale bar* shown in **b** is 20 μm

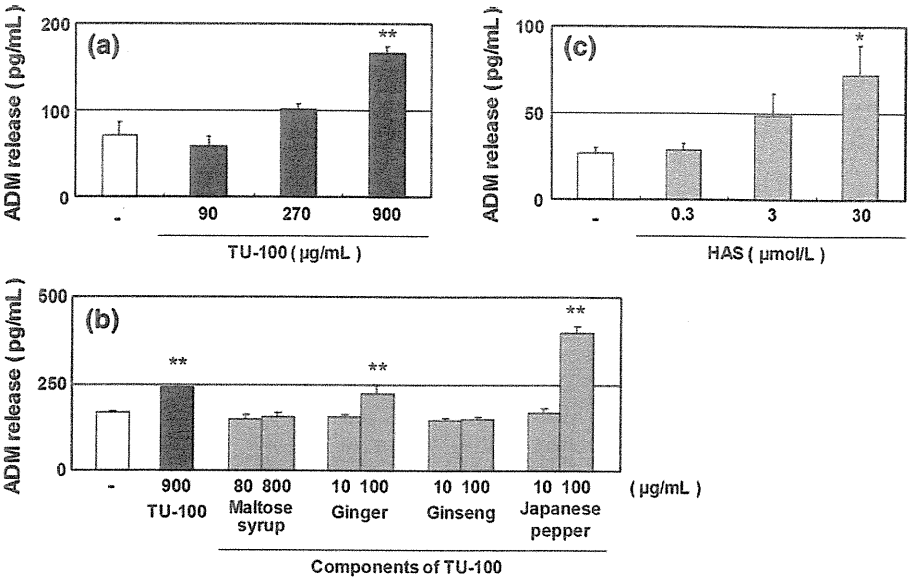

Fig. 7 Effects of TU-100 and its components on ADM release by rat epithelial cell line. **a** TU-100 was evaluated at various concentrations. **b** Maltose syrup powder, processed ginger, ginseng radix, and Japanese pepper were evaluated at the concentrations indicated in the figure. **c** Hydroxy α-sanshool (*HAS*) was evaluated at various concentrations. The concentration of ADM in each sample was measured using an EIA kit. $N = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control, respectively (Dunnett's test)

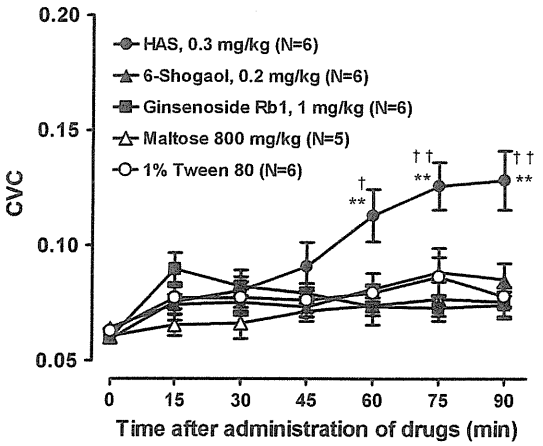

Fig. 8 Effects of specific constituents of TU-100 on colonic blood flow in colitis rats. HAS, ginsenoside Rb1, 6-shogaol, and maltose were evaluated at the respective doses contained in 900 mg/kg of TU-100. The vehicle (1% Tween 80) was also evaluated. $N = 5$–6 each. Factorial two-way ANOVA revealed significant effects of group [$F(4, 144) = 21.17$ $p < 0.0001$] and time [$F(5, 144) = 3.95$, $p = 0.0022$]. $^{**}p < 0.01$ versus pre-administration (0 min) (Dunnett's test). $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus the vehicle control, respectively (Student's *t*-test)

TNBS-induced colitis rats than in control rats, while ADM was unchanged at the protein and mRNA levels (Fig. 5). It has been reported that the CGRP concentrations in the colons of patients with CD were decreased compared with that of the normal colon [7]. We demonstrated that the βCGRP mRNA expression was 23.4 times that of αCGRP mRNA in the colon of control rats, and that the gene expression of βCGRP, but not αCGRP, was decreased in ischemic segments of the TNBS-treated colon. Therefore, the decrease of the CGRP concentration in the CD intestine may be related to the decrease of βCGRP, which is expressed in intrinsic primary afferent neurons [24, 26]. Previous observations of several structural and functional abnormalities of the enteric nervous system in patients with CD support our findings [35, 36].

As we previously reported, TU-100 increased colonic blood flow in normal rats via up-regulation of the CGRP system [19]. The present study using the colons of TNBS rats, however, suggests that the increase of blood flow induced by TU-100 may be mediated predominantly via the ADM system (Fig. 3). Further, in the present TNBS model, TU-100 did not increase the expression of CGRP receptors, which was reported to be upregulated in normal rat intestines [19]. Thus, it seems that the CGRP system is heavily damaged in CD [7, 27] and in TNBS-instilled intestines. Activation of the ADM system, which appears to be intact even in TNBS-instilled intestines (Fig. 5), may therefore be a better therapeutic option than the restoration/reinforcement of a damaged CGRP system.

In the present study, ADM immunoreactivity was observed in the mucosal epithelium and in the crypts of small and large intestines (Fig. 6). We and other researchers have observed similar results in various species, including humans [17, 37–40]. TU-100 was administered directly into the colon in the present study, and therefore it is reasonable to consider that TU-100 may induce the release and/or production of ADM by directly interacting with intestinal epithelial cells. Based on the above assumption, we screened several intestinal epithelial cell lines for ADM production and found that IEC-6, a non-transformed intestinal epithelial cell line derived from the rat intestinal crypt, produced ADM. IEC-6 is derived from the small intestine; however, we have observed that the intraduodenal administration of TU-100 increased blood flow in the jejunum and that an antibody to ADM abolished the vasodilatory effect of TU-100 (unpublished observation). Further, we have