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緩和医療学

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放射線科医がはじめた緩和医療

本家好文

はじめに

「放射線治療を担当する医者は，どうせ放射線の“かけ屋”だ．患者からの苦情を聞くのは自分たちだし，治療方針には口を挟まないで欲しい」．約25年前に，ある医師からいわれた言葉です．いまでも忘れられない言葉ですが，筆者が「チーム医療」が重要なことを実感し，ベッドサイドで「患者さんの声に耳を傾けることの大切さ」を認識させられた言葉でもあります．

ベッドサイドで患者さんの声を聴きつづけたことから，痛みをとることの大切さを学び，放射線治療医から緩和医療を専門とする医師に転身したといっても過言ではありません．

放射線治療医として

「何もしないわけにはいかないし，放射線でもかけておくか」．これも放射線治療医時代の自分にとっては，忘れられない言葉です．放射線治療には臓器の形態を保って機能が温存できることや，身体への負担が小さいといったメリットがあります．喉頭癌や舌癌では手術よりも放射線治療で機能を温存することで，患者さんのQOL（quality of life）が維持されることはよく知られています．

最近では，乳癌治療で乳房温存手術と放射線を用いることによって乳房を温存し，美容面や精神面でよい結果が残せるようになりました．機能が温存できるだけでなく，負担の軽い放射線治療の役割は，今後ますます大きくなることが予測されています．

しかし，一般的には放射線治療というと「副作用が強い」ことばかりが強調されて，十分に活用されていないのが現状です．ひと昔前までは，手術ができない患者さんに「仕方なく」実施することや，再発や転移巣への治療を依頼されることが多かったのです．

骨転移の痛みに対しては，放射線治療をおこなうことによって身体が動かせるようになったり，オピオイド鎮痛剤を減量できるといったメリットがあります．しかし，以前には治癒が望めない状態で「痛みをとるためだけ」の治療に対して，放射線治療医も「姑息的放射線治療」と称して，あまり関心をもってきませんでした．最近になって苦痛の緩和を目的とする「緩和的放射線治療」の大切さが，ようやく理解されるようになりました．

大学病院から第一線病院へ

卒業して3年目からの2年間，放射線医学総合研究所（放医研：千葉市）で放射線治療の基

本家好文（Honke Yoshifumi）/広島県緩和ケア支援センター

礎を学ぶ機会を得ました．その後，広島に戻ってからの5年間は地域のがん治療医に放射線治療を正しく理解してもらい，手術療法や化学療法と連携して集学的治療を実践することに力を注ぎました．不治の病といわれていた「がん」を放射線で治すことにエネルギーを注いだ時期でもありました．

卒業して10年目にあたる1985年に，厚生連広島総合病院に新しく放射線治療部門が設立されて赴任しました．放射線治療医は患者さんを診ないといわれたことへの反発心から，広島総合病院では放射線治療中の患者さんは自らが主治医となって治療をおこないました．

大学病院の放射線治療部門では，完全に治癒できる可能性のある患者さんも数多くおられました．しかし，第一線病院では8～9割の患者さんたちは，紹介された当初から治癒が望めない進行がんという状況でした．

大学病院時代には，患者さんやご家族の声が届きにくい立場にいましたが，再び第一線病院に勤務することになり，主治医として直接患者さんやご家族の声を聴く機会が増えました．がんに罹患したことによる不安や恐怖だけでなく，痛みが改善しないことに対する辛い気持ちを毎日聴くようになりました．

その当時から，患者さんのところにうかがうときには，必ず腰をかけて座って話し合うように心がけました．最初は照れ臭くて抵抗がありましたが，じっくり患者さんの話を「聴く」ことは，患者さんに大変喜ばれたのでいまでもつづけています．

疼痛治療の重要性

多くの医師は，自分の将来の方向性を左右するような忘れられない患者さんとの出会いを体験しています．筆者にとっては15年前に出会った50歳代の乳癌患者さんとの出会いが，緩和医療を志すきっかけになりました．

まだ硫酸モルヒネ徐放剤が発売されて間もないころ，自分自身のモルヒネ使用方法に関する知識が未熟で経験が不足していたために，十分な量のモルヒネを使わず「痛みと向き合う毎日」を余儀なくさせてしまいました．その結果，最終的には病棟から投身自殺をされてしまい，スタッフも自分自身も大きなショックを受けました．その患者さんの体験をきっかけにして，病棟内で医師・看護師・薬剤師とで疼痛治療の勉強会をはじめました．勉強会を通じて学ぶことによって，徐々に痛みを抱えた患者さんへの治療が上手くいくようになりました．その後，疼痛治療の勉強会を病棟から病院全体の「院内ターミナルケア研究会」に発展させていきました．

病院内部で研究会を開催することによって，疼痛治療に対する病院内の医療者の意識が大きくかわりました．モルヒネの具体的な使用方法を学ぶことによって，病院全体のがん性疼痛治療のレベルが明らかに改善しました．

病院から地域へ

さらに広島県内の医療機関にも声をかけて「ターミナルケアを考える会・広島」を発足させました．この会は，自分にとって緩和医療をめざす基盤となる研究会となっています．発足以来10年以上が経過していますが，「継続は力なり」の言葉を信じていまもつづけています．会

の活動は地域のメディアにも注目されるようになり，社会的な支援を受けたことも大きな励みになりました．

「ターミナルケアを考える会・広島」がスタートした1993年には，ホスピスをみたことがありませんでした．そこでデーケン氏（元上智大学）の主催するヨーロッパのホスピス視察ツアーに参加して，はじめて英国のホスピス施設を見学して基本的な考え方に接したり，全国から集まった人たちとの意見交換ができたことや，英国の大学医学部で「緩和医療学」が講座として確立していることを知ったことなどが，自分を「緩和医療」に向かわせる大きな刺激になりました．

放射線治療と緩和医療の両立

緩和ケアへの関心が高まるにつれて，逆に放射線治療への関心が徐々に薄れていく自分を感じていました．緩和医療と放射線治療とを両立させるむずかしさを悩んでいたときに，国立呉病院（現：独立行政法人国立病院機構呉医療センター）に緩和ケア病棟が開設され，担当医師を探しているという話が舞い込みました．放射線治療医として全身の悪性腫瘍にかかわってきた25年間の経験を生かしながら，緩和医療を専門にする医師に転身することを決意しました．

2000年1月からは国立呉病院緩和ケア病棟に勤務しました．一般病棟に勤務しているときには，医師と看護師が患者さんのケアについて10分間のカンファレンスをもつことも簡単ではありませんでしたが，緩和ケア病棟ではカンファレンスを開催できることが当たり前という状況でした．痛みを緩和するためにはどんなアプローチが必要か，鎮痛剤は有効か，副作用の問題は生じていないか，身体的痛み以外の問題を抱えていないかといったことを話し合っていると，チーム医療を実践していることを実感できました．

しかし一方で，緩和ケア病棟に勤務していると，一般病棟の感覚とのずれを感じることもあって戸惑いもありました．緩和ケア病棟に入院するのだから，積極的な治療をおこなうことは認めないといった雰囲気を感じることもありました．患者さんの心理状態を考えると，自分が積極的ながん治療が困難で緩和ケアの対象となる病状であることは，説明を受けて理屈では理解していても，何とかならないだろうかという期待感をもっていることも多いのです．そのことを認めないような姿勢で入院の判断をすることもあり反省させられました．

現在のがん医療では，積極的ながん治療の効果が得にくくなった時期の患者さんへの援助が欠落しているように感じます．緩和ケア病棟や在宅ケアという選択をする前段階の患者さんで，将来の方向性について一番迷っている時期の患者さんたちへの支援が必要だと思います．そのような時期の患者さんに対して緩和医療がもっとかかわる必要性があると感じています．

広島県緩和ケア支援センター

最近の5年間に広島県に8つの緩和ケア病棟が整備されました．最も新しく2004年9月に開設したばかりの広島県緩和ケア支援センターでは，県内8番目の緩和ケア病棟の運用だけでなく，広島県全体の緩和ケアの推進を目標とした緩和ケア支援室の運用をおこなっています．

緩和ケア支援室の事業としては，地域で独自に緩和ケアを担う人材を育成するための教育研修事業，患者さんや医療関係者から直接相談を受ける電話相談や面談窓口，また直接県内の各

地域との連携を図り具体的な援助をおこなうアドバイザー派遣事業や，在宅緩和ケアを推進するための「デイホスピス」事業などをおこなっています．

少し長期的な展望で，地域の在宅緩和ケアを中心とした緩和ケアの推進に取り組んでいく予定です．

おわりに

緩和医療は医療の分野ではいぜんとしてマイナーな分野で，決して十分な理解が得られているとはいえない状況にあります．しかし，徐々に関心が高まっていることも事実です．今後とも，一人ひとりの患者さんを苦痛から解放することを積み重ねながら，緩和医療の重要性について啓発活動をつづけていきます．

Cellular/Molecular

Protease-Activated Receptor-1 and Platelet-Derived Growth Factor in Spinal Cord Neurons Are Implicated in Neuropathic Pain after Nerve Injury

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Recently, it has been reported that both thrombin-sensitive protease-activated receptor 1 (PAR-1) and platelet-derived growth factor (PDGF) are present not only in platelets, but also in the CNS, which indicates that they have various physiological functions. In this study, we evaluated whether PAR-1/PDGF in the spinal cord could contribute to the development of a neuropathic pain-like state in mice. Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were significantly suppressed by repeated intrathecal injection of hirudin, which is characterized as a specific and potent thrombin inhibitor. Furthermore, a single intrathecal injection of thrombin produced long-lasting hyperalgesia and allodynia, and these effects were also inhibited by hirudin in normal mice. In nerve-ligated mice, the increase in the binding of [^{35}S]GTPγS to membranes of the spinal cord induced by thrombin and PAR-1-like immunoreactivity (IR) in the spinal cord were each greater than those in sham-operated mice. Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were also suppressed by repeated intrathecal injection of either the PDGF α receptor (PDGFRα)/Fc chimera protein or the PDGFR-dependent tyrosine kinase inhibitor AG17 [(3,5-di-tert-butyl-4-hydroxybenzylidene)-malononitrile]. Moreover, thermal hyperalgesia and tactile allodynia induced by thrombin in normal mice were virtually eliminated by intrathecal pretreatment with PDGFRα/Fc. In immunohistochemical studies, PAR-1-like IR-positive cells in the spinal dorsal horn were mostly colocated on PDGF-like IR-positive neuronal cells. These data provide novel evidence that PAR-1 and PDGF-A-mediated signaling pathway within spinal cord neurons may be directly implicated in neuropathic pain after nerve injury in mice.

Key words: thrombin; protease-activated receptor-1; platelet-derived growth factor; neuropathic pain; spinal cord; pain

Introduction

Recently, our understanding of the role of serine proteases such as thrombin, a key enzyme of the coagulation system, has been expanded to include actions in the nervous system (Gill et al., 1998). Thrombin affects protease-activated receptors (PARs), which are a family of G-protein-coupled receptors (Macfarlane et al., 2001). Molecular cloning has identified four PARs. Thrombin activates PAR-1, PAR-3, and PAR-4. Thrombin cleaves PARs, unmasking the "tethered ligand," an extracellular N-terminal domain that subsequently binds and activates the receptor (Steinhoff et al., 2000). It is considered that PARs are implicated in responses to injury, notably in inflammation and repair (Dai et al., 2004). In particular, PAR-1, which mediates most of the known proinflammatory actions of thrombin, is expressed by platelets, endothelial cells, fibroblasts, smooth muscle cells, mast cells, neurons, and astrocytes (Suo et al., 2004). Furthermore, PAR-1 is present in the developing and mature CNS (Fang et al., 2003). Zhu et al. (2005) reported that PAR-1 is expressed not only in a subset of large-diameter primary sensory neurons but also in a subset of small- and medium-diameter primary sensory neurons of the spinal cord, which play an important role in the transmission of pain. The thrombin inhibitor hirdin, which is isolated from leech (*Hirudo medicinalis*), was characterized as a specific thrombin inhibitor in the late 1950s (Markwardt, 1957). In addition, the high affinity of hirudin for thrombin and the specificity of the tight and essentially irreversible binding of hirudin to thrombin has been confirmed recently (Markwardt, 2002). Therefore, hirudin is a useful tool for the elucidation of mechanisms of thrombin-induced physiological responses.

Recently, it has been reported that thrombin induces the activation of platelets and promotes the expression or the release of platelet-derived growth factor (PDGF) from α-granule of platelets (Fager, 1995). PDGF was identified 20 years ago as a growth-promoting activity in human platelets for fibroblasts, smooth muscle cells, and glial cells. There are three PDGF isoforms (PDGF-AA, PDGF-AB, and PDGF-BB), which are homodimers or heterodimers of related A and B polypeptide chains. Two re-

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ceptors for PDGF [PDGF α receptor (PDGFRα) and PDGFRβ] bind the PDGF isoforms with different affinities. The PDGFRα binds all three PDGF isoforms with similar affinities. The binding of PDGF to PDGFR has been shown to induce homodimerization of the receptor, which facilitates the intrinsic protein tyrosine kinase (PTK) activity of PDGFR (Claesson, 1994). The activation of PTK autophosphorylates several tyrosine residues found within the cytoplasmic domain of PDGFR and provides a recognition site for intracellular signal molecules. Furthermore, it has been reported that PDGF and PDGFR could be located in myelinated and unmyelinated primary sensory neurons (Eccleston et al., 1993) and in the spinal cord (Heldin and Westermark, 1999). Thus, these findings support the idea that PDGF, which is mostly present in the blood vessels, may play a more important role in the physiological responses including pain perception than has been thought previously. In addition, as described above, thrombin could release PDGF through PAR in platelets. Together, these findings raise the possibility that PDGF associated with PAR may be implicated in pain perception. However, the contribution of a thrombin/PAR/PDGF pathway to the neuropathic pain-like state is unknown.

In the present study, therefore, we investigated the role of a spinal thrombin/PAR/PDGF-mediated signaling pathway in the development of the neuropathic pain-like state induced by partial sciatic nerve ligation in mice.

Materials and Methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the number and suffering of any animals used in the following experiments. Animals were used only once in the present study. All behavioral experiments were conducted in a single-blind manner to avoid the effect of subjectivity.

Animals. Male Institute of Cancer Research mice (Tokyo Laboratory Animals Science, Tokyo, Japan) weighing ~25 g were housed at a room temperature of 23 ± 1°C with a 12 h light/dark cycle (light on 8:00 A.M. to 8:00 P.M.). Food and water available *ad libitum* during the experimental period.

Neuropathic pain model. The mice were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). We produced a partial sciatic nerve injury by tying a tight ligature with 7-0 or 8-0 silk suture around approximately one-third to one-half of the diameter of the sciatic nerve on the right side (ipsilateral side) under a light microscope (SD30; Olympus, Tokyo, Japan) as described previously (Malmberg et al., 1997). In sham-operated mice, the nerve was exposed without ligation.

Measurement of the latency of paw withdrawal in response to a thermal stimulus. To assess the sensitivity to thermal stimulation, each of the hindpaws of mice was tested individually using a thermal stimulus apparatus (model 33 Analgesia Meter; IITC Life Science, Woodland Hills, CA). The intensity of the thermal stimulus was adjusted to achieve an average baseline paw withdrawal latency of ~8–10 s in naive mice. Only quick hindpaw movements (with or without licking of hindpaws) away from the stimulus were considered to be a withdrawal response. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured alternating between the left and right with an interval of >3 min between measurements. The latency of paw withdrawal after the thermal stimulus was determined as the average of three measurements per paw. Before the behavioral responses to the thermal stimulus were tested, mice were habituated for at least 1 h in an acrylic cylinder (15 cm in height and 8 cm in diameter). Under these conditions, the latency of paw withdrawal in response to the thermal stimulus was tested. The latency of paw withdrawal in response to a thermal stimulus was measured before surgery and 1, 3, 5, 7, and 14 d after surgery.

To investigate the effect of a single intrathecal treatment with thrombin or PDGF in normal mice, the latency of paw withdrawal was measured before and after injection until the latency returned to the baseline. The latency of paw withdrawal in response to a thermal stimulus was determined as the average of both paws.

Measurement of paw withdrawal in response to a tactile stimulus. To quantify the sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured using von Frey filaments (North Coast Medical, Morgan Hill, CA) with two different bending forces (0.02 and 0.16 g). Each von Frey filament was applied to the plantar surface of the hindpaws for 3 s, and this was repeated three times. Each of the hindpaws of the mice was tested individually. Paw withdrawal in response to a tactile stimulus was evaluated by scoring as follows: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. Paw withdrawal in response to each filament was determined as the average of two scores per paw. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured alternating between left and right with an interval of >3 min between the measurements. Before the behavioral responses to a tactile stimulus were tested, mice were habituated for at least 1 h on an elevated nylon mesh floor. Under these conditions, paw withdrawal in response to a tactile stimulus was tested. The paw withdrawal threshold to the tactile stimulus was measured before surgery and the day after measurement of the thermal threshold (day 2, 4, 6, 8, and 15). To investigate the effect of a single intrathecal treatment with thrombin or PDGF in normal mice, the measurement of paw withdrawal in responses to the tactile stimulus was performed before and after the injection until the response returned to the baseline. The paw withdrawal in response to the tactile stimulus was determined as the average of both paws.

Intrathecal injection. Intrathecal injection was performed as described by Hylden and Wilcox (1980) using a 25 μl Hamilton syringe with a 30 one-half gauge needle. The needle was inserted into the intervertebral space between L5 and L6 of the spinal cord. A reflexive flick of the tail was considered to be a sign of the accuracy of each injection. The injection volume was 4 μl for intrathecal injection. In the present study, intrathecal injection in mice was performed under unanesthesia.

Groups of mice were repeatedly treated intrathecally with the thrombin inhibitor hirudin (30 pmol/mouse), a recombinant mouse PDGFRα/Fc chimera protein (10 ng/mouse; PDGFRα/Fc) or the PDGF receptor-dependent PTK inhibitor (3,5-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile (AG17) (30 nmol/mouse) 30 min, 1 h, or 30 min, respectively, before surgery and once per day for 8 consecutive days after surgery. A single intrathecal injection of thrombin (1 pmol/mouse) or a recombinant human PDGF (0.1 pmol/mouse; PDGF) was performed in naive mice. Intrathecal pretreatment with hirudin, AG17, or PDGFRα/Fc was performed 30 min, 30 min, or 1 h, respectively, before a single intrathecal injection of thrombin or PDGF.

[^{35}S]GTPγS binding assay. Seven days after nerve ligation or sham operation, for membrane preparation, spinal cords were rapidly excised at 4°C. The tissue was homogenized using a Potter-Elvejham tissue grinder with a Teflon pestle in 20 vol (w/v) of ice-cold Tris-HCl buffer containing (in mM): 50 Tris-HCl, pH 7.4, 5 $MgCl_2$, and 1 EGTA. The homogenate was centrifuged at 4°C for 10 min at 48,000 × g. The pellet was resuspended in [^{35}S]GTPγS binding assay buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4°C for 10 min at 48,000 × g. The final pellet was resuspended in [^{35}S]GTPγS binding assay buffer and stored at −70°C until use. The membrane homogenate (3–8 μg protein/assay) was incubated at 25°C for 2 h in 1 ml of assay buffer with various concentrations of thrombin (0.001–10 μM; Wako, Osaka, Japan), 30 μM GDP and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham Biosciences, Arlington Heights, IL). The reaction was terminated by filtration using a Brandel cell harvester (Model M-24; Brandel, Gaithersburg, MD) and Whatman (Ann Arbor, MI) GF/B glass filters that had been presoaked in 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, transferred to scintillation-counting vials con-

taining 0.5 ml of a tissue solubilizer (Soluene-350; PerkinElmer, Boston, MA) and 4 ml of a scintillation cocktail (Hionic Fluor; PerkinElmer), and equilibrated for 12 h, and the radioactivity in the samples was determined with a liquid scintillation analyzer. Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The binding of [^{35}S]GTPγS was measured in the presence of GDP and the absence of agonist. The data are expressed as the mean ± SEM for percentage stimulation. Similar results were obtained from at least three independent sets of experiments.

Sample preparation. Seven days after nerve ligation or sham operation, mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and intracardially perfusion fixed with freshly prepared 4% paraformaldehyde in 0.1 M PBS, pH 7.4. After perfusion, the lumbar spinal cord was quickly removed and postfixed in 4% paraformaldehyde for 2 h, and then permeated with 20% sucrose in 0.1 M PBS for 1 d and 30% sucrose in 0.1 M PBS for 2 d with agitation. The L5 lumbar spinal cord segments were then frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at −30°C until use. Frozen spinal cord segments were cut with a freezing cryostat (Leica CM 1510; Leica, Wetzlar, Germany) at a thickness of 10 μm and thaw mounted on poly-L-lysine-coated glass slides.

Immunohistochemistry. The spinal cord sections were blocked in 10% normal goat serum (NGS) in 0.01 M PBS for 1 h at room temperature. Each primary antibody [1:50–150 thrombin R (PAR-1; Santa Cruz Biotechnology, Santa Cruz, CA), 1:3000 protein kinase Cγ (PKCγ; Santa Cruz Biotechnology), 1:120 PDGF-A (Santa Cruz Biotechnology), 1:400 neuronal nuclei (NeuN; Chemicon, Temecula, CA), 1:1000 S100β-subunit clone SH-B1 (S100β; Sigma, St. Louis, MO)] was diluted in 0.01 M PBS containing 10% NGS and incubated for two nights at 4°C. The samples were then rinsed and incubated with an appropriate secondary antibody conjugated with Alexa 488 and Alexa 546 for 2 h at room temperature. Because the staining intensity might vary between experiments, control sections were included in each run of staining. The slides were then coverslipped with PermaFluor aqueous mounting medium (Immunon; Thermo Electron, Pittsburgh, PA). All sections were observed with a light microscope (Olympus BX-80) and photographed with a digital camera (CoolSNAP HQ; Olympus).

RNA preparation and quantitative analysis by reverse transcription-PCR. Total RNA in the spinal cord of thrombin- or PBS-injected mice was extracted using the SV Total RNA Isolation system (Promega, Madison, WI) following the manufacturer's instructions. Purified total RNA was quantified spectrophotometrically at A_{260}. To prepare first-strand cDNA, 1 μg of RNA was incubated in 100 μl of buffer containing 10 mM dithiothreitol, 2.5 mM $MgCl_2$, dNTP mixture, 200 U of reverse transcriptase II (Invitrogen, Carlsbad, CA), and 0.1 mM oligo-dT_{12-18} (Invitrogen). The PDGF-A gene was amplified in a 50 μl PCR solution containing 0.8 mM $MgCl_2$, dNTP mixture, and DNA polymerase, with synthesized primers: a sense primer of PDGF-A, which is at position 407–423 (5′-CTGTGCCCATTCGCAGG-3′) of the PDGF-A and an antisense primer at position 915–929 (5′-ACCGCACGCACATTG-3′), which was designed according to GenBank sequence accession number AY324648. Samples were heated to 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The final incubation was 72°C for 7 min. The mixture was run on 2% agarose gel electrophoresis with the indicated markers and primers for the internal standard glyceraldehyde-3-phosphate dehydrogenase. The agarose gel was stained with ethidium bromide and photographed with UV transillumination. The intensity of the bands was analyzed and semiquantified by computer-assisted densitometry using NIH Image software. Values represent the mean ± SEM of three independent experiments.

Drugs. The drugs used in the present study were thrombin (Wako); a thrombin inhibitor, hirudin (Sigma); PDGFRα/Fc (R & D Systems, Minneapolis, MN); a PDGF receptor-dependent PTK inhibitor, AG17 (Calbiochem, La Jolla, CA); and PDGF (R & D Systems). Thrombin and PDGFRα/Fc were dissolved in sterile PBS, pH 7.4. Hirudin was dissolved in 0.9% sterile physiological saline. AG17 was dissolved in 0.9% sterile physiological saline containing 6% dimethylsulfoxide. PDGF was dissolved in 0.1% bovine serum albumin containing 4 mM HCl.

Statistical analysis. All data are presented as the mean ± SEM. The statistical significance of differences between groups was assessed with two-way ANOVA or one-way ANOVA followed by the Bonferroni/Dunn multiple-comparison test or with Student's *t* test.

Results

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation in mice were significantly suppressed by repeated intrathecal injection of the thrombin inhibitor hirudin

Partial ligation of the sciatic nerve caused a marked decrease in the latency of paw withdrawal against a thermal stimulus only on the ipsilateral side in nerve-ligated mice [vs Saline-Sham group, $F_{(1,7)} = 168.441$, $p < 0.001$ (Fig. 1*A*)]. The thermal hyperalgesia observed on the ipsilateral side in nerve-ligated mice was significantly suppressed by repeated intrathecal injection of hirudin (30 pmol/mouse) just before ligation and once per day for 8 consecutive days after nerve ligation [vs Saline-Ligation group, $F_{(1,9)} = 108.923$, $p < 0.001$ (Fig. 1*A*)].

Mice with sciatic nerve ligation also showed a marked increase in paw withdrawal in response to a tactile stimulus only on the ipsilateral side in nerve-ligated mice [vs Saline-Sham group, $F_{(1,7)} = 69.829$, $p < 0.001$ (Fig. 1*B*); vs Saline-Sham group, $F_{(1,7)} = 124.623$, $p < 0.001$ (Fig. 1*C*)]. Under these conditions, repeated intrathecal injection of hirudin (30 pmol/mouse) significantly suppressed the increase in paw withdrawal in response to an innocuous tactile stimulus induced by nerve ligation in mice [vs Saline-Ligation group, $F_{(1,9)} = 40.565$, $p < 0.001$ (Fig. 1*B*); vs Saline-Ligation group, $F_{(1,9)} = 37.352$, $p < 0.001$ (Fig. 1*C*)]. In addition, the recovery of the persistent reduction in the thermal threshold by hirudin lasted even after treatment with hirudin was terminated (from day 9 to day 15) [vs Saline-Ligation group, $F_{(1,9)} = 34.934$, $p < 0.001$ (Fig. 1*B*); vs Saline-Ligation group, $F_{(1,9)} = 51.586$, $p < 0.001$ (Fig. 1*C*)].

Repeated intrathecal injection of hirudin at the doses used in the present study failed to affect thermal and tactile thresholds on the contralateral side in nerve-ligated mice and on both sides in sham-operated mice (data not shown).

[^{35}S]GTPγS binding by the PAR agonist thrombin to membranes of the spinal cord was significantly increased in nerve-ligated mice

The ability of the endogenous PAR ligand thrombin to activate G-proteins in the spinal cord of sham-operated or nerve-ligated mice was examined by monitoring the binding of [^{35}S]GTPγS to spinal cord membranes. Thrombin (0.001–10 μM) produced a concentration-dependent increase in the binding of [^{35}S]GTPγS to the L5 lumbar spinal cord membranes obtained from sham-operated mice. In membranes of the spinal cord from nerve-ligated mice, the increase in [^{35}S]GTPγS binding induced by thrombin was significantly greater than that obtained from sham-operated mice ($F_{(1,90)} = 141.7$; $p < 0.001$) (Fig. 2).

PAR-1-like immunoreactivity in the dorsal horn of the mouse spinal cord was increased by sciatic nerve ligation

PAR-1-like immunoreactivity (IR) was detected on the ipsilateral side of the L5 lumbar spinal dorsal horn of sham-operated mice (Fig. 3*A*). Seven days after sciatic nerve ligation, PAR-1-

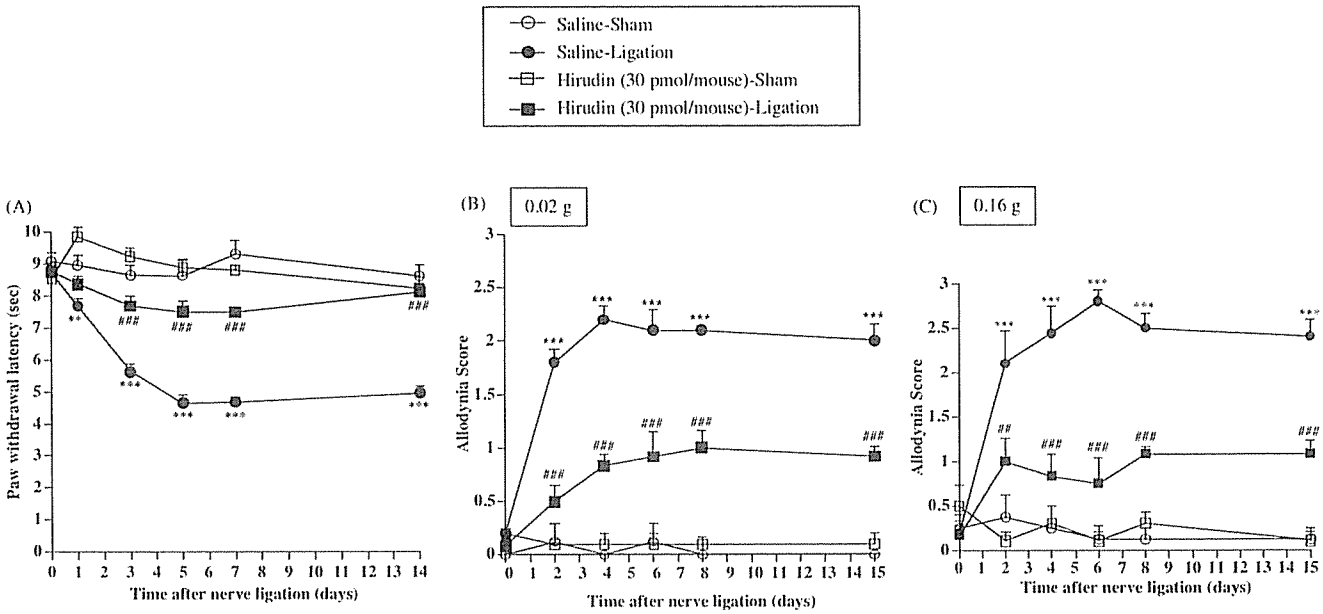

Figure 1. *A–C*, Effect of repeated intrathecal injection of a thrombin inhibitor, hirudin, on thermal hyperalgesia (*A*) and tactile allodynia (*B*, *C*) in sham-operated or nerve-ligated mice. Tactile stimulus was applied using filaments with two different bending forces [0.02 g (*B*) and 0.16 g (*C*)]. sec, Seconds. Groups of mice were repeatedly treated intrathecally with hirudin (30 pmol/mouse) or saline 30 min before surgery (day 0) and once per day for 8 consecutive days after surgery. From day 9 to day 15 after surgery, mice were not treated with hirudin. Each point represents the mean ± SEM of five to seven mice. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus Saline-Sham group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus Saline-Ligation group. Error bars represent SEM.

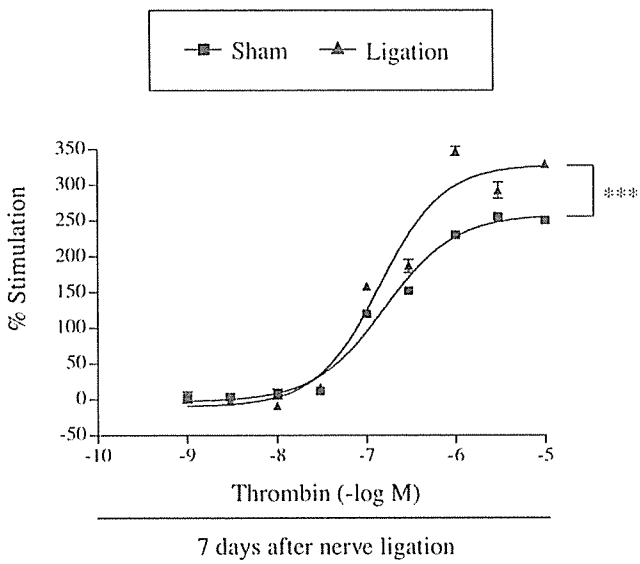

Figure 2. Comparison of the stimulation of [^{35}S]GTPγS binding by the PAR agonist thrombin to membranes of the spinal cord obtained from sham-operated or nerve-ligated mice. Sample preparation was performed at 7 d after nerve ligation. The spinal cord membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with or without thrombin. The data are shown as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and the absence of thrombin. Values represent the mean ± SEM of three samples. $F_{(1,90)} = 141.7$ and $p < 0.001$ versus Sham group. Error bars represent SEM.

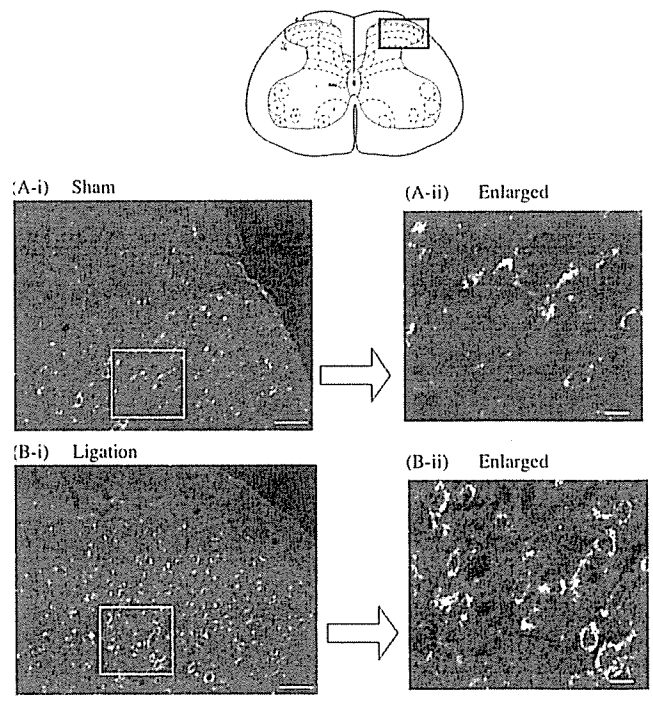

Figure 3. Increase in PAR-1-like IR on the ipsilateral superficial dorsal horn of the L5 lumbar spinal cord in nerve-ligated mice (*B-i*, *B-ii*) compared with that in sham-operated mice (*A-i*, *A-ii*). Spinal cord slices were prepared 7 d after sham operation or nerve ligation in mice. Scale bars: *A-i*, *B-i*, 50 μm; *A-ii*, *B-ii*, 10 μm.

like IR in lamina II and III of the ipsilateral side of the L5 lumbar spinal dorsal horn was increased compared with that observed in sham-operated mice (Fig. 3*B*). Furthermore, the increased PAR-1-like IR in the spinal cord after nerve injury was colocalized with PKCγ, which is highly limited to neuronal cells in the inner part of lamina II of the dorsal horn of the spinal cord (Fig. 4).

Thermal hyperalgesia and tactile allodynia induced by a single intrathecal treatment with thrombin in normal mice were suppressed by intrathecal pretreatment with hirudin

We next investigated whether exogenous intrathecal treatment with thrombin could cause a hyperalgesic or allodynic response in normal mice. A single intrathecal injection of

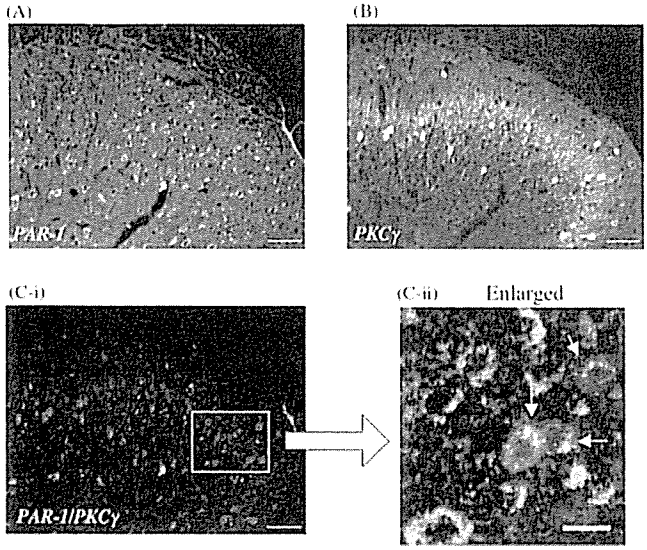

Figure 4. *A, B,* Colocalization of PAR-1 (*A*) with PKCγ, which is highly limited to the inner part of lamina II in the dorsal horn of the mouse spinal cord (*B*). *C-i, C-ii,* The green labeling for PAR-1 and the red labeling for PKCγ show colocalization in the dorsal horn of the spinal cord. PAR-1-like IR is seen in the membrane of PKCγ-labeled cells in the dorsal horn of the spinal cord (arrowhead in *C-ii*). Scale bars: *A, B, C-i,* 50 μm; *C-ii,* 10 μm.

thrombin (1 pmol/mouse) produced marked thermal hyperalgesia and tactile allodynia in normal mice after injection, and these effects lasted for 9–10 d after injection [vs Saline-PBS group, $F_{(1,10)} = 605.881$, $p < 0.001$ (Fig. 5*A*); vs Saline-PBS group, $F_{(1,10)} = 138.103$, $p < 0.001$ (Fig. 5*B*); vs Saline-PBS group, $F_{(1,10)} = 247.557$, $p < 0.001$ (Fig. 5*C*)]. The long-lasting thermal hyperalgesia and tactile allodynia caused by an exogenous single intrathecal injection of thrombin were abolished by intrathecal pretreatment with the thrombin inhibitor hirudin (30 pmol/mouse) [vs Saline-Thrombin group, $F_{(1,10)} = 818.014$, $p < 0.001$ (Fig. 5*A*); vs Saline-Thrombin group, $F_{(1,10)} = 81.366$, $p < 0.001$ (Fig. 5*B*); vs Saline-Thrombin group, $F_{(1,10)} = 111.593$, $p < 0.001$ (Fig. 5*C*)].

PDGF-A-like immunoreactivity was detected in the neuron and astrocytes in the mouse spinal cord

We investigated the localization of PDGF-A in the L5 lumbar spinal cord in mice using immunohistochemical analysis. PDGF-A-like IR was detected in the superficial laminas and inner part of the L5 lumbar spinal cord. Furthermore, PDGF-A-like IR was principally colocalized with the neuron-specific nuclear protein marker NeuN-positive cells in the superficial and inner part of the dorsal horn. Astrocytes in the dorsal horn of the spinal cord were stained with S100β, a specific marker for astrocytes. PDGF-A-like IR was observed occasionally in S100β-positive cells in the dorsal horn of the spinal cord (Fig. 6).

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation in mice were suppressed by repeated intrathecal injection of PDGFRα/Fc

Repeated intrathecal injection of PDGFRα/Fc (10 ng/mouse) just before and once per day for 8 consecutive days after nerve ligation reversed the decreased thermal and tactile threshold on the ipsilateral side of nerve-ligated mice [vs PBS-Ligation group, $F_{(1,10)} = 179.88$, $p < 0.001$ (Fig. 7*A*); vs PBS-Ligation group, $F_{(1,10)} = 68.049$, $p < 0.001$ (Fig. 7*B*); vs PBS-Ligation group, $F_{(1,10)} = 47.454$, $p < 0.001$ (Fig. 7*C*)]. The same treatment had no effect on the latency of paw withdrawal after a thermal or tactile stimulus on the contralateral side in nerve-ligated mice and on both sides in sham-operated mice (data not shown).

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were significantly reversed by repeated intrathecal injection of the PDGF receptor-dependent PTK inhibitor AG17

To investigate whether the activation of PTK mediated by PDGF receptor could be directly involved in the neuropathic

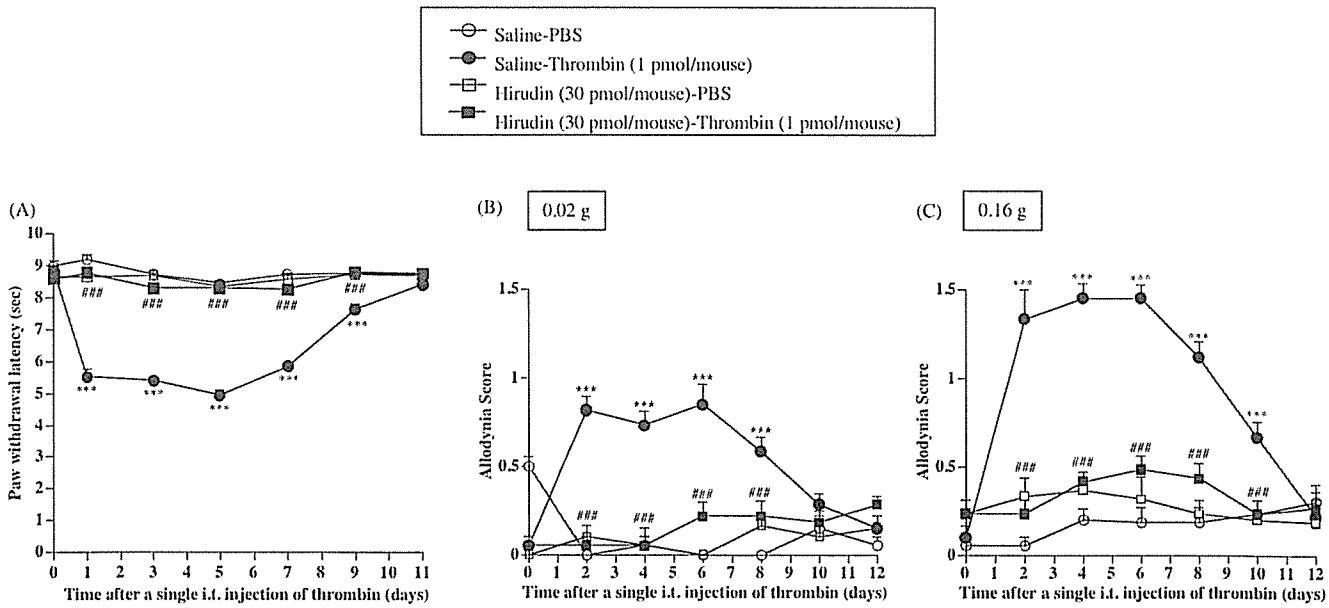

Figure 5. *A–C,* Effect of a single intrathecal injection of thrombin on paw withdrawal responses to the thermal (*A*) and tactile (*B, C*) stimulus in normal mine. sec, Seconds. *B, C,* Tactile stimulus was performed using filament with two different bending forces [0.02 g (*B*) and 0.16 g (*C*)]. Groups of mice were repeatedly treated intrathecally with a thrombin inhibitor hirudin (30 pmol/mouse) or saline 30 min before a single intrathecal injection of thrombin and once per day for 12 consecutive days after injection. Each point represents the mean ± SEM of six mice. $^{***}p < 0.001$ versus Saline-PBS group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus Saline-Thrombin group. i.t., Intrathecal. Error bars represent SEM.

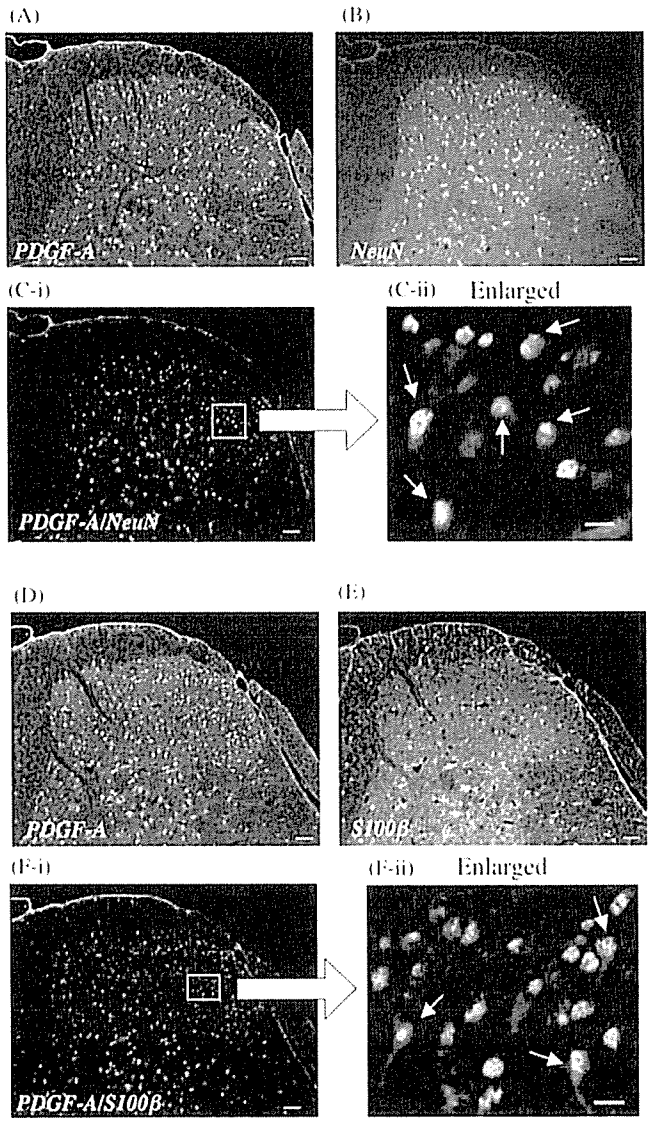

Figure 6. Colocalization of PDGF-A (*A*, *D*) with NeuN (*B*), a neuron-specific nuclear protein marker, or S100β (*E*), a specific marker for astrocytes, in the dorsal horn of the mouse spinal cord. The green labeling for PDGF-A and the red labeling for NeuN or S100β show colocalization in the dorsal horn of the spinal cord (*C-i*, *C-ii*, or *F-i*, *F-ii*). PDGF-A-like IR is exclusively observed on NeuN-labeled cells (*C-ii*) and partially observed on S100β-labeled cells in the dorsal horn of the spinal cord (*F-ii*). Scale bars: *A*, *B*, *C-i*, *D*, *E*, *F-i*, 50 μm; *C-ii*, *F-ii*, 10 μm.

pain-like state induced by nerve ligation, groups of mice were treated intrathecally with the PDGF receptor-dependent PTK inhibitor AG17 (30 nmol/mouse) just before surgery and once per day for 8 consecutive days after nerve ligation. The thermal hyperalgesia and tactile allodynia induced by nerve ligation were significantly reversed by chronic intrathecal treatment with AG17 [vs Vehicle-Ligation group, $F_{(1,11)} = 116.508$, $p < 0.001$ (Fig. 8*A*); vs Vehicle-Ligation group, $F_{(1,11)} = 20.928$, $p < 0.001$ (Fig. 8*B*); vs Vehicle-Ligation group, $F_{(1,11)} = 25.063$, $p < 0.001$ (Fig. 8*C*)]. Such treatment had no effect on the latency of paw withdrawal on the contralateral side in nerve-ligated mice or on both sides in sham-operated mice (data not shown).

Thermal hyperalgesia and tactile allodynia induced by a single intrathecal treatment with PDGF in normal mice were suppressed by intrathecal pretreatment with AG17

To investigate whether PDGF could cause a hyperalgesic or allodynic response in normal mice, groups of normal mice were treated intrathecally with PDGF. A single intrathecal injection of PDGF (0.1 pmol/mouse) produced marked thermal hyperalgesia and tactile allodynia in normal mice after injection, and this effect lasted for 8–9 d after injection [vs Vehicle-PBS group, $F_{(1,8)} = 164.250$, $p < 0.001$ (Fig. 9*A*); vs Vehicle-PBS group, $F_{(1,8)} = 197.951$, $p < 0.001$ (Fig. 9*B*); vs Vehicle-PBS group, $F_{(1,8)} = 93.523$, $p < 0.001$ (Fig. 9*C*)]. The long-lasting thermal hyperalgesia and tactile allodynia caused by an exogenous single intrathecal injection of PDGF were abolished by intrathecal pretreatment with the PDGF receptor-dependent PTK inhibitor AG17 (30 nmol/mouse) [vs Vehicle-PDGF group, $F_{(1,8)} = 376.449$, $p < 0.001$ (Fig. 9*A*); vs Vehicle-PDGF group, $F_{(1,8)} = 49.965$, $p < 0.001$ (Fig. 9*B*); vs Vehicle-PDGF group, $F_{(1,8)} = 68.029$, $p < 0.001$ (Fig. 9*C*)].

Thermal hyperalgesia and tactile allodynia induced by a single intrathecal treatment with thrombin in normal mice were significantly eliminated by repeated intrathecal pretreatment with PDGFRα/Fc

Facilitation of the activation of a thrombin/PAR-1/PDGF pathway in the spinal cord may play an important role in the development of the neuropathic pain-like state in mice. The subsequent study was undertaken to investigate whether spinal PDGF could be involved in the thermal hyperalgesia or tactile allodynia observed with a single intrathecal injection of thrombin. The thermal hyperalgesia and tactile allodynia induced by a single intrathecal injection of thrombin were significantly eliminated by repeated intrathecal pretreatment with PDGFRα/Fc just before injection and once per day for 12 consecutive days after injection [vs PBS-Thrombin group, $F_{(1,9)} = 463.624$, $p < 0.001$ (Fig. 10*A*); vs PBS-Thrombin group, $F_{(1,9)} = 38.222$, $p < 0.001$ (Fig. 10*B*); vs PBS-Thrombin group, $F_{(1,9)} = 70.964$, $p < 0.001$ (Fig. 10*C*)]. Under these conditions, there were no differences between PBS- and thrombin-injected mice with regard to mRNA levels of PDGF-A in the spinal cord (Fig. 11).

PAR-1-positive cells were clearly colocalized with PDGF-A-like immunoreactivity in the dorsal horn of the spinal cord of nerve-ligated mice

The key approach in the present study was to investigate the colocalization of PAR-1 with PDGF-A in the dorsal horn of the spinal cord of nerve-ligated mouse. Interestingly, almost all of the PAR-1-positive cells in the dorsal horn of the spinal cord of nerve-ligated mice were clearly colocalized with PDGF-A-like IR, indicating that PDGF-A-like IR is highly located on PAR-1-positive neuronal cells (Fig. 12).

Discussion

A very large number of studies have confirmed that a serine protease, thrombin, is a major stimulus for platelets, and initiates a series of coordinated events that result in platelet aggregation *in vitro* or *in vivo* (Macfarlane et al., 2001). In addition to its well known role in the platelet aggregation cascade, thrombin has been implicated in degenerative as well as protective mechanisms in the CNS. It is of interest to note that PAR-1, one of the thrombin receptor subtypes, is expressed in a subset of small and medium diameter of primary sensory neurons, suggesting that a thrombin/PAR-1 pathway plays a possible role in the transmission of pain (Zhu et al., 2005). It has been reported that responses to thrombin were found to be blocked by the specific thrombin inhibitor hirudin, a hirudin-derived peptide, which reflects the thrombin-specific nature of the cloned receptor. Several studies

have demonstrated that the response to thrombin is unrelated to the proteolytic properties of the enzyme, but rather the hirudin-like binding domain appears to be the important feature of the protein. Mutation of the N terminus identified the presence of a hirudin-like domain that was essential for high-affinity binding and the potent effects of thrombin (Macfarlane et al., 2001). The hirudin-like thrombin binding sequence has been identified in PAR-1. This would perhaps allow high-affinity binding to PAR-1 in the hirudin-like binding domain. In the present study, we first investigated the role of a spinal thrombin/PAR-1 pathway in the development of a neuropathic pain-like state in mice. Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were significantly suppressed by intrathecal pretreatment with hirudin just before and once per day for the first 7 d after surgery. Interestingly, the recovery of the persistent reduction in the thermal threshold by hirudin lasted even after treatment with hirudin was discontinued, which indicates the importance of the initial blockade of PAR-1 for inhibiting the development of a neuropathic pain-like state. In our preliminary study, we also investigated whether the thrombin/PAR-1 pathway within the spinal cord could be involved in the maintenance, as well as the development, of a neuropathic pain-like state in mice. The developed neuropathic pain-like state was significantly suppressed even by repeated intrathecal posttreatment with hirudin, which was started from 7 d after sciatic nerve ligation (thermal hyperalgesia, 66.4 ± 2.68% of maximum inhibition; tactile allodynia, 82.5 ± 7.85% of maximum inhibition). These results suggest that the thrombin/PAR-1 pathway within the spinal cord may also contribute to the maintenance of a neuropathic pain-like state. Furthermore, we demonstrated here that an exogenous single intrathecal injection of thrombin produced long-lasting thermal hyperalgesia and tactile allodynia in normal mice. These responses were abolished by intrathecal pretreatment with hirudin. These findings indicate that the spinal thrombin/PAR-1 pathway may be directly involved in the development of a neuropathic pain-like state caused by nerve ligation in mice.

Additional evidence for the contribution of a spinal thrombin/PAR-1 pathway to a neuropathic pain-like state was obtained by an [^{35}S]GTPγS binding assay and immunohistochemical approach. Possible changes in PAR-1 func-

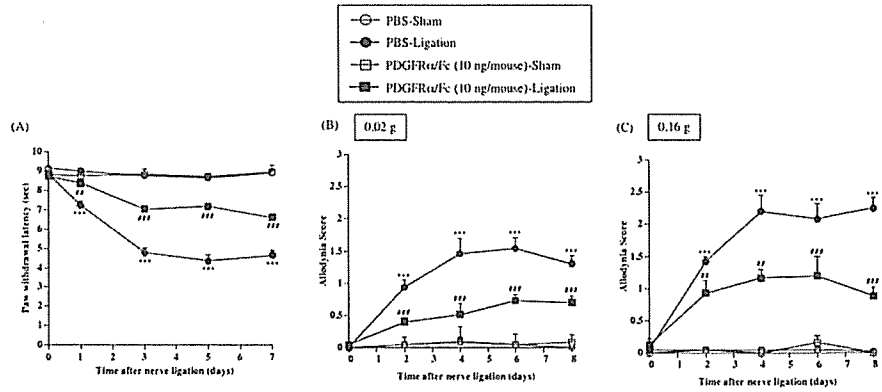

Figure 7. *A–C*, Effect of repeated intrathecal injection of PDGFRα/Fc on thermal hyperalgesia (*A*) and tactile allodynia (*B*, *C*) in sham-operated or nerve-ligated mice. sec, Seconds. *B*, *C*, Tactile stimulus was applied using filaments with two different bending forces [0.02 g (*B*) and 0.16 g (*C*)]. Groups of mice were repeatedly treated intrathecally with PDGFRα/Fc (10 ng/mouse) or sterile PBS 1 h before surgery (day 0) and once per day for 8 consecutive days after surgery. Each point represents the mean ± SEM of five to six mice. $^{***}p < 0.001$ versus PBS-Sham group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus PBS-Ligation group. Error bars represent SEM.

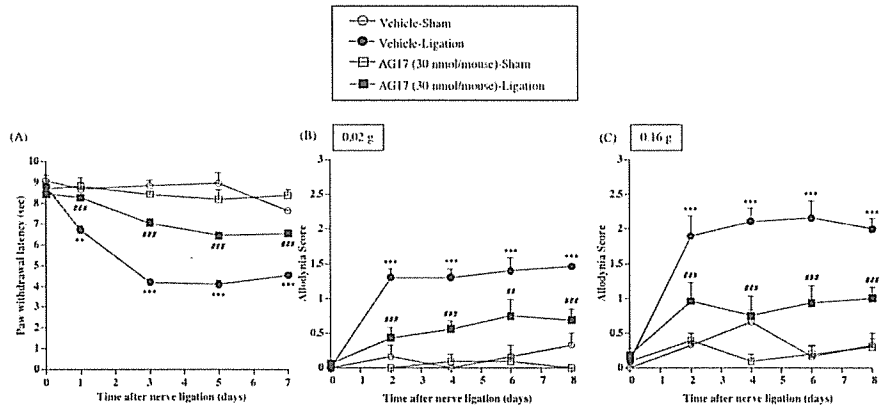

Figure 8. *A–C*, Effect of repeated intrathecal injection of an inhibitor of PDGF receptor-mediated PTK, AG17, on thermal hyperalgesia (*A*) and tactile allodynia (*B*, *C*) in sham-operated or nerve-ligated mice. sec, Seconds. *B*, *C*, Tactile stimulus was applied using filaments with two different bending forces [0.02 g (*B*) and 0.16 g (*C*)]. Groups of mice were repeatedly treated intrathecally with AG17 (30 nmol/mouse) or vehicle 30 min before surgery (day 0) and once per day for 8 consecutive days after surgery. Each point represents the mean ± SEM of five to seven mice. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus Vehicle-Sham group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus Vehicle-Ligation group. Error bars represent SEM.

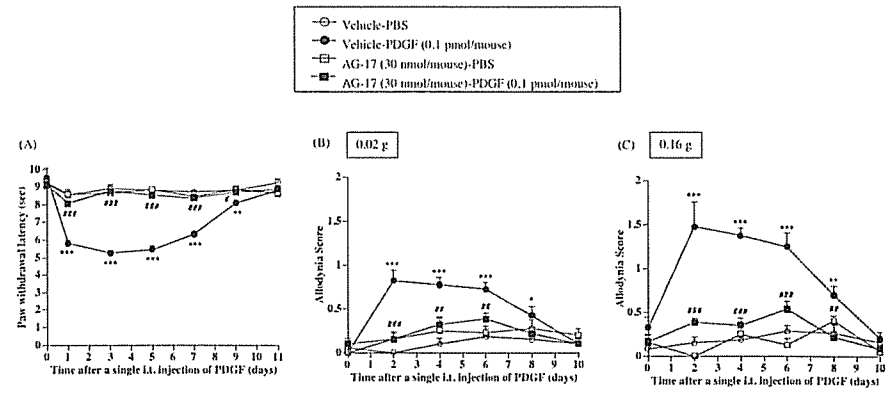

Figure 9. *A–C*, Effect of a single intrathecal injection of PDGF on paw withdrawal responses to the thermal (*A*) and tactile (*B*, *C*) stimulus in normal mice. sec, Seconds. *B*, *C*, Tactile stimulus was performed using filament with two different bending forces [0.02 g (*B*) and 0.16 g (*C*)]. Groups of mice were repeatedly treated intrathecally with an inhibitor of PDGF receptor-mediated PTK, AG17 (30 nmol/mouse), or vehicle 30 min before a single intrathecal injection of PDGF and once per day for 11 consecutive days after injection. i.t., Intrathecal. Each point represents the mean ± SEM of six mice. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus Vehicle-PBS group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus Vehicle-PDGF group. Error bars represent SEM.

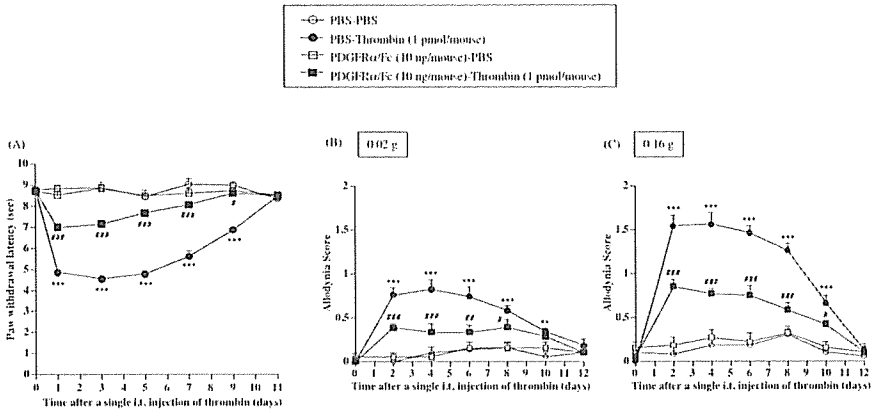

Figure 10. Effect of repeated intrathecal injection of PDGFRα/Fc on thermal hyperalgesia or tactile allodynia induced by a single intrathecal injection of thrombin in normal mice. Groups of mice were repeatedly treated intrathecally with PDGFRα/Fc (10 ng/mouse) or sterile PBS 1 h before the single intrathecal injection of thrombin (1 pmol/mouse) and once per day for 12 consecutive days after injection. Each point indicates the mean ± SEM of six mice. sec, Seconds; i.t., intrathecal. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus PBS-PBS group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus PBS-Thrombin group. Error bars represent SEM.

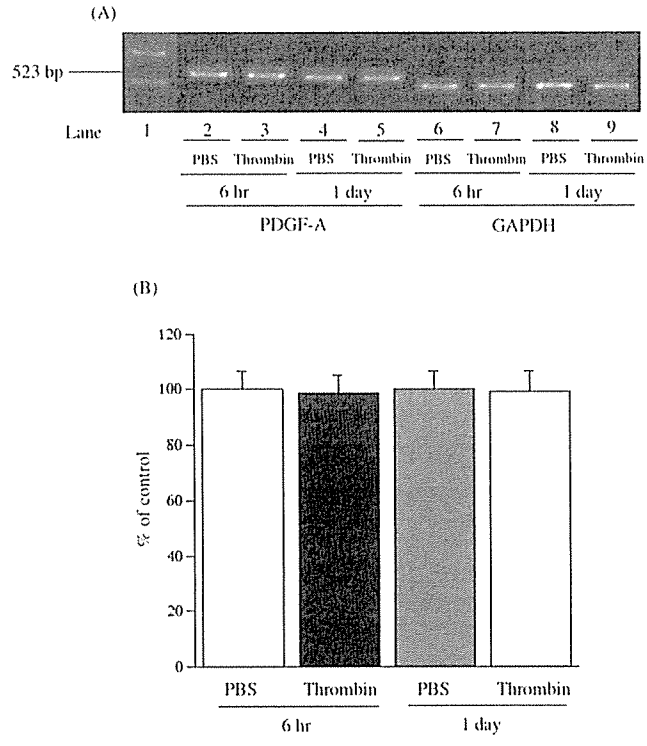

Figure 11. *A*, Representative reverse transcription-PCR for PDGF-A (lanes 2–5) and an internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH; lanes 6–9) mRNAs in the spinal cord at 6 h and 1 d after a single intrathecal injection of PBS (lanes 2, 4, 6, and 8) or thrombin (1 pmol/mouse; lanes 3, 5, 7, and 9) in mice. Lane 1 is the indicated marker. *B*, The intensity of the bands was determined in a semiquantitative manner using NIH Image software. Each value for PDGF-A mRNA in thrombin-injected mice was normalized by the value for the respective GAPDH mRNA. The values in thrombin-injected mice are expressed as a percentage of the increase in PBS-injected mice. Each column represents the mean ± SEM of 10 samples. Error bars represent SEM.

tion in the spinal cord of nerve-ligated mice were evaluated by monitoring the binding of [^{35}S]GTPγS to membranes of the mouse spinal cord. In membranes of the L5 lumbar spinal cord from nerve-ligated mice, the concentration-dependence curve for the increased [^{35}S]GTPγS binding induced by thrombin was significantly greater than that obtained in sham-operated mice. Furthermore, an immunohistochemical study showed that sciatic nerve ligation produced a clear increase in PAR-1-like IR on lamina II and III of the ipsilateral side of the L5 lumbar spinal dorsal horn compared with that observed in sham-operated mice. The subpopulations of PAR-1-like IR in the spinal cord were colabeled with PKCγ-like IR, which is highly limited to neuronal cells in the inner part of lamina II of the dorsal horn of the spinal cord, indicating that PAR-1 in the dorsal horn of the L5 lumbar spinal cord may be mostly located in neurons. These findings provide additional evidence that a thrombin/PAR-1 pathway within neuronal cells of the L5 lumbar spinal cord plays a critical role in the development of a neuropathic pain-like state.

PDGF was first identified in a search for serum factors that stimulate the proliferation of arterial smooth muscle cells (Hoch and Soriano, 2003). Although the α-granule of platelets is a major storage site for PDGF, recent studies have shown that PDGF can be synthesized by several different cell types (Heldin and Westermark, 1999). In the present study, PDGF-A-like IR was detected in the superficial laminas and inner part of the L5 lumbar spinal cord in mice. Furthermore, PDGF-A-like IR in the spinal cord was predominantly located on neuronal cells and occasionally located on astrocytes. Under these conditions, we found here for the first time that thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were significantly suppressed by repeated intrathecal treatment with PDGFRα/Fc, which can trap an endogenous PDGF, resulting in the prevention of receptor activation. Furthermore, a single intrathecal injection of PDGF produced marked thermal hyperalgesia and tactile allodynia in normal mice after injection, and these effects lasted for 8–9 d after injection. These responses were abolished by intrathecal pretreatment with a potent inhibitor of PTK activity linked to PDGF receptors, AG17. Thermal hyperalgesia and tactile allodynin observed on the ipsilateral side in nerve-ligated mice was abolished by repeated intrathecal injection of AG17. Considering these findings, the present data suggest that the release of PDGF within the spinal cord by nerve ligation may lead to the development of a neuropathic pain-like state in mice.

Recently, thrombin has been shown to activate platelets and promote the expression or the release of PDGF from platelets. It has also been suggested that the activation of thrombin receptor leads to several intracellular signaling events as well as to the stimulation of endogenous PDGF-A production (Fager, 1995). In the present study, we demonstrated here that PDGF-A-like IR was detected in the neuronal cells and astrocytes within the L5 lumbar spinal cord. It is of interest to note that PDGF-A-like IR was colocalized with PAR-1 in the dorsal horn of the L5 lumbar spinal cord. Together, these findings raise the fascinating possibility that the activation of PAR-1 by thrombin may facilitate the expression or the release of PDGF in neuronal cells and astrocytes located in the spinal cord.

The subsequent study was then undertaken to investigate whether spinal PDGF could be involved in thermal hyperalge-

sia or tactile allodynia mediated through the thrombin/PAR-1 pathway. The thermal hyperalgesia and tactile allodynia induced by a single intrathecal injection of thrombin were significantly eliminated by repeated intrathecal pretreatment with PDGFRα/Fc just before injection and once per day for 12 consecutive days after injection. Under these conditions, there were no differences between PBS- and thrombin-injected mice with regard to mRNA levels of PDGF-A in the spinal cord. These results suggest that thrombin may activate PDGF-A-containing fibers in the spinal cord without changing the expression of PDGF-A mRNAs, resulting in the induction of hyperalgesia and allodynia.

In a subsequent study, we investigated the colocalization of PAR-1-containing cells with PDGF-A in the dorsal horn of the spinal cord of nerve-ligated mice. Interestingly, almost all of the PAR-1-like IR-positive cells in the dorsal horn of the spinal cord of nerve-ligated mice were clearly colabeled with PDGF-A-like IR, indicating that PDGF-A is mostly located on PAR-1-containing neuronal cells.

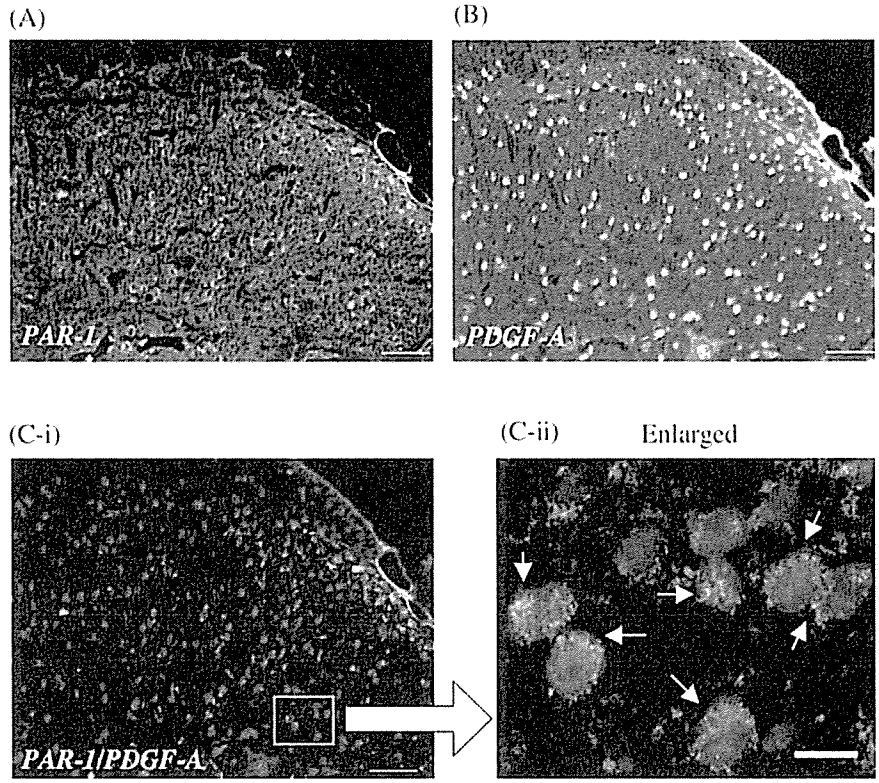

Figure 12. *A*, *B*, Colocalization of PAR-1 (*A*) with PDGF-A (*B*) in the dorsal horn of the mouse spinal cord. ***C-i***, ***C-ii***, The green labeling for PAR-1 and the red labeling for PDGF-A show apparent colocalization in the dorsal horn of the spinal cord. PDGF-A-like IR is almost all seen in the cytoplasm of PAR-1-labeled cells in the dorsal horn of the spinal cord (***C-ii***, arrow). Scale bars: ***A***, ***B***, ***C-i***, 50 μm; ***C-ii***, 10 μm.

Released PDGF acts on PDGF receptor. It is well recognized that the activation of PDGF receptor and its autophosphorylation serve as docking sites for adapter proteins and enzymes such as phosphoinositide-3 kinase, phospholipase Cγ (PLCγ), and the Src family of tyrosine kinases (Heldin and Westermark, 1999). In particular, the activation of PLCγ triggers the facilitation of PKC, which involves in the development of a neuropathic pain-like state (Ohsawa et al., 2000; Zou et al., 2004), through the production of diacylglycerol and inositol 1,4,5-triphosphate. Furthermore, brain-derived neurotrophic factor, which was observed almost entirely on presynaptic neurons of the L5 lumbar mouse spinal cord, is an important modulator of the expression of neurotrophin associated with the activation of PKC (Mannion et al., 1999; Yajima et al., 2005). However, we found here that PDGF-A-positive cells are predominantly located on postsynaptic neurons and are widely expressed in the outer and inner parts of the laminas of the spinal cord in mice. These findings suggest that spinal PDGF may be more essential for the development of the neuropathic pain-like state induced by nerve ligation in mice. Although we clearly show here that both PAR-1 and PDGF are responsible for this type of the neuropathic pain-like state, we cannot completely exclude the possibility that these two components are just necessary for the present model to be developed. Thus, additional investigation is needed to identify the role of PAR-1 and PDGF in other types of chronic pain models.

In conclusion, the present data provide novel evidence that thrombin/PAR-1 and PDGF-A-mediated signaling pathway within the spinal cord are directly involved in the development of the neuropathic pain-like state induced by sciatic nerve ligation in mice. Such findings raise the fascinating possibility that the activation of upregulated PAR-1 located on the spinal dorsal horn neurons activated by thrombin released after nerve injury may release PDGF in the spinal cord and in turn activate PDGFRα, leading to a neuropathic pain-like state.

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Effect of a selective $GABA_B$ receptor agonist baclofen on the μ-opioid receptor agonist-induced antinociceptive, emetic and rewarding effects

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Abstract

The management of excessive adverse effects of opioids is a major clinical problem. The present study was undertaken to investigate the effect of a selective γ-aminobutyric acid $(GABA)_B$ receptor agonist baclofen on the μ-opioid receptor agonist-induced antinociceptive, emetic and rewarding effects. Either morphine or fentanyl produced a dose-dependent antinociceptive effect in both ferrets using Randall-Selitto test and mice using tail-flick test. Under these conditions, pretreatment of baclofen produced an additive antinociception induced by morphine or fentanyl. Furthermore, the augmentation of antinociception induced by systemic administration of baclofen with morphine or fentanyl was completely abolished by either i.c.v. or i.t. pretreatment with the selective $GABA_B$ receptor antagonist CGP 35348 in mice. We next investigated the emetic response induced by μ-opioid receptor agonist in ferrets. Morphine at lower doses than that used for antinociceptive assay produced both retching and vomiting, whereas fentanyl failed to produce the retching and vomiting in ferrets. Here we reported for the first time that baclofen significantly suppressed the retching and vomiting induced by morphine, indicating the involvement of $GABA_B$ receptor in emetic control pathway. Furthermore, baclofen also inhibited place preference elicited morphine or fentanyl in rats. Taken together, these results suggest that co-administration of baclofen with μ-opioid receptor agonist produced a potentiation of antinociceptive effect, whereas an untoward effect was completely blocked.

Keywords: Emesis; Antinociception; Rewarding effect; Baclofen; Ferret

1. Introduction

According to the World Health Organization (WHO) guidelines for patients with moderate or severe pain, morphine has been a "gold standard" for treatment of moderate to severe cancer pain. However, the use of morphine for the treatment of cancer pain is sometimes accompanied by side effects such as emesis, constipation and drowsiness. The management of excessive adverse effects of opioids is considered to be a major clinical

Abbreviations: DMN, dorsal motor nucleus; GABA, γ-aminobutyric acid; CGP 35348, (3-Aminopropyl)(diethoxymetyl)phosphinic acid; CNS, central nervous system; DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin; DVC, dorsal vagal complex; i.c.v., intracerebroventricular; i.t., intrathecal; mPfc, medial prefrontal cortex; VTA, ventral tegmental area.

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problem. Therefore, the detailed understanding of opioid-related side effects and the strategies used to prevent and manage them are essential skills for pain management.

Fentanyl is one series of potent μ-opioids and used for opioid rotation in the clinic and exhibits 50–100 times more potent analgesic activity than that of morphine. It is believed that side effects are fewer than those of morphine (Cherny et al., 2001).

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS) and has been implicated in the regulation of many physiological processes. Three types of GABA receptors, i.e. $GABA_A$, $GABA_B$ and $GABA_C$ have been distinguished on the basis of distinct pharmacological and physiological properties (Macdonald and Olsen, 1994; Misgeld et al., 1995; Johnston, 1996). $GABA_B$ receptor belongs to the superfamily of G protein-coupled receptors and it shows the highest sequence homology to metabotropic glutamate receptors (Kaupmann et al., 1997). Stimulation of $GABA_B$ receptors in cell bodies and dendrites elicits slow inhibitory postsynaptic potentials, and their activation at presynaptic nerve terminals inhibits neurotransmitter release (Misgeld et al., 1995).

Considerable progress has recently developed highly specific $GABA_B$ receptor agonists and antagonists, and these drugs have been explored in a number of animal models. Clinically, baclofen, a selective $GABA_B$ receptor agonist, is available and used to treat migraine headache, muscosceletal pain, and pain associated with trigeminal neuralgia, stroke and spinal cord injury (Fromm, 1994; Taira et al., 1995; Loubser and Akman, 1996; Hering-Hanit, 1999; Becker et al., 2000). The effectiveness of clinical use of baclofen is further supported by several reports that baclofen also reduces the reinforcing effects of drugs of abuse including opioids in human and animal models (Cousins et al., 2002).

The present study was undertaken to examine the effect of baclofen on the morphine-induced antinociceptive, emetic and rewarding effects, in order to further investigate the advantages for treatment of baclofen combined with opioids for the pain management.

2. Methods

The present study was conducted in accordance with Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments.

2.1. Animals

Male ferrets weighing 1.0–1.5 kg were obtained from Marshall Research Labs (New York, USA). Male SD rats weighing 250–300 g and male ICR mice weighing 20–25 g were obtained from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). Animals were housed in a room maintained at 23 ± 1 °C under a 12-h light/dark cycle (light on 08:00–20:00 hours). Food and water were available ad libitum.

2.2. Assessment of antinociceptive response for ferrets (Randall-Selitto test)

The antinociceptive effects induced by morphine and fentanyl were measured using Randall-Selitto test analgesy-meter (Muromachi Kikai Co., Ltd., Tokyo, Japan). The pressure was directly applied to the tail of the ferret via a negative-shape plunger. The tail withdrawal or vocalization was considered to be a nociceptive response. The pressure threshold was shown in grams and the cut-off threshold was set at 350 g. Ferrets were administered subcutaneously (s.c.) with morphine or fentanyl, and the measurement of antinociception induced by these drugs were performed every 15 min after the injection. The antinociceptive effect was calculated as a percentage of maximum possible effect (%Antinociception) according to the following formula: %Antinociception = (test pressure threshold − predrug pressure threshold) × 100/(cut-off pressure threshold − predrug pressure threshold). To investigate the effect of baclofen, groups of ferrets were pretreated with baclofen 30 min before injection of μ-opioid receptor agonist.

2.3. Antinociceptive assay for mice (tail-flick test)

The antinociceptive response produced by morphine or fentanyl was evaluated by recording the tail-flick test (Tail-Flick Analgesia Meter Model MK 300B, Muromachi Kikai Co., Ltd., Tokyo, Japan). To prevent tissue damage, we established a 10-s cut-off time. The tail-flick latency was measured both before and after the challenge with morphine or fentanyl. Antinociceptive response was calculated as a percentage of maximum possible effect (%Antinociception) according to the following formula: %Antinociception = (test latency − predrug latency)100/(cut-off time − predrug latency). The treatment with baclofen (3 mg/kg) for the morphine- or fentanyl-induced antinociception was conducted 30 min before each injection in mice.

2.4. Rota-rod assay for mice

Motor incoordination produced by baclofen in mice was evaluated by the time until fall-off from a rota-rod (3 cm in diameter) at 8 rpm (KN-95; Natsume Seisakusyo, Co., Ltd., Tokyo, Japan). First of all, mice were trained to be able to walk on the rota-rod for 60 s. This training was performed for 3 days. Next day of the final training,

the time until fall-off from the rota-rod was measured up to 60 s at 30 min after the baclofen (1, 3 and 5.6 mg/kg, s.c.) injection.

2.5. Intracerebroventricular injection

Intracerebroventricular (i.c.v.) administration was performed following the method described previously (Cherny et al., 2001). The injection was made with a 2-mm double-needle (Natsume Seisakusho, Co., Ltd., Tokyo) attached to a 25-μl Hamilton microsyringe. Solution was injected in a volume of 4 μl per mouse. Groups of mice were pretreated i.c.v. with the selective $GABA_B$ receptor antagonist (3-aminopropyl)(diethoxymethyl)phosphinic acid (CGP 35348, 1.7 nmol/mouse) 10 min prior to baclofen (3 mg/kg, s.c.) injection.

2.6. Intrathecal injection

Intrathecal (i.t.) administration was performed following the method described previously (Cousins et al., 2002) using a 25-μl Hamilton syringe with a 30-gauge needle. Solution was injected in a volume of 4 μl per mouse. Groups of mice were pretreated i.t. with CGP 35348 (1.7 nmol/mouse) 10 min prior to baclofen (3 mg/kg, s.c.) injection.

2.7. Evaluation of emetic response

Before the measurement of emesis induced by morphine and fentanyl, ferrets were acclimatized for 30 min in individual cages. The emetic response was evaluated by counting the number of retching or vomiting for 30 min after the drug injection. Retching was defined as any rhythmic abdominal contraction without expulsion, whereas vomiting was defined as any oral expulsion (solid or liquid) from gastrointestinal tract. An assessment of emesis was made over 30 min beginning with morphine or fentanyl injection. To determine the effect of baclofen on the morphine-induced emesis, groups of ferrets were pretreated with baclofen 30 min before morphine injection.

2.8. Conditioned place preference test

Place conditioning was performed according to our previous report (Suzuki, 1996). The apparatus consisted of a shuttle box (30 × 60 × 30 cm: $w \times l \times h$) that is divided into two compartments of equal size. One compartment is white with a textured floor and the other is black with a smooth floor. For conditioning, rats were confined to one compartment after drug injection and the other compartment after saline injection for 1 h. The order of the injection (drug or saline) and the compartment (white or black) were counter balanced across the subjects.

Conditioning sessions were conducted once daily for six days (three days for drug, three days for saline). Immediately after s.c. injection of morphine or fentanyl, animals were placed in one compartment for 1 h. On alternate days, animals receiving with saline were placed in the other compartment for 1 h. Baclofen was administered 30 min before each conditioning. On day 7, test conditioning was performed as follows: the partition separating two compartments was raised to 12 cm above the floor, and the neutral platform is inserted along the seam separating the compartments. The rats, which had been treated with neither drugs nor saline on day 7, were placed on the platform. The time spent in each compartment during a 900-s test session was then recorded automatically in blinded fashion using an infrared beam sensor (KN-80, Natsume Seisakusyo Co., Ltd., Tokyo, Japan). All sessions were conducted under the condition of dim illumination (28 lx lamp) and white masking noise.

2.9. Drugs

The drugs used in the present study were morphine hydrochloride (Sankyo Co., Tokyo, Japan), fentanyl citrate (a gift kindly from Hisamitsu Pharmaceutical Co., Inc., Tokyo, Japan), (±) baclofen (Sigma Chemical Co, St. Louis, MO, USA) and CGP 35348 (Tocris Cookson Ltd., United Kingdom). All drugs were dissolved in saline (Otsuka Pharmaceutical Co., Inc., Tokyo, Japan).

2.10. Statistical analysis

The data are represented as the mean ± S.E.M. The statistical significance of differences between the groups was assessed with a two-way ANOVA, followed by Bonferroni/Dunn.

3. Results

3.1. Antinociceptive effect of μ-opioid receptor agonist in the ferret Randall-Selitto test

Either morphine (1.5, 3 and 6 mg/kg, s.c.) or fentanyl (10, 30 and 56 μg/kg, s.c.) produced a dose-dependent antinociception in the ferret Randall-Selitto test. The peak antinociceptive response induced by morphine or fentanyl reached at 30 or 15 min after the injection, respectively ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. saline group, Fig. 1A, B). The ED_{50} value for the