Table 3 Training methods used for practical training

実習項目	実習形式
歯科麻酔に関する医療面接	見学，座学，相互，シミュレーション実習
麻酔管理計画	シミュレーション実習
バイタルサインの測定	自験，相互実習
注射法	相互実習，シミュレーション実習
一次救命処置	シミュレーション実習
二次救命処置	シミュレーション実習，座学実習
器具を用いた気道確保法	シミュレーション実習
浸潤麻酔	自験，相互実習，シミュレーション実習
伝達麻酔	相互実習，シミュレーション実習
モニタリングによる全身管理	見学
笑気吸入鎮静法	相互実習
静脈内鎮静法	見学
全身麻酔法	見学，シミュレーション実習
その他	相互実習，見学

学生教育においては昭和59年の歯科麻酔学教授要綱に全身管理の項目が初めて記載され[6]，それ以後，歯科麻酔学教育において重要な項目となった．歯科医療においても全身管理の重要性については広く認識されるようになってきており，全身管理が可能な歯科医師の養成には歯科麻酔学の卒前教育は不可欠となっている．また，歯科医師の麻酔科研修や救急研修においても歯科麻酔学の卒前教育が十分に行われていることが前提になっている[2]．

しかし，前述したように全国の歯学部・歯科大学における歯科麻酔学の卒前教育に関する本格的な調査はこれまでに行われたことがなかった．今回，日本歯科麻酔学会卒前教育ワーキンググループでアンケート形式での実態調査を行うことになり，各大学の歯科麻酔学卒前教育担当者の協力を得て有用な知見を得ることができた．

講義に関しては項目では大学間に大きな差は認められず，コア・カリキュラムの項目はほとんど講義されていた．各大学で採用している歯科麻酔学の教科書は何種類かあり必ずしも同一ではないが，項目に関してはどの教科書もほぼ同じであり，ほとんどの項目の講義が行われていた．ただ小児，障害者，高齢者の麻酔管理に関する講義を単独の項目として行っていない大学もあった．小児歯科，障害者歯科，高齢者歯科などで行われている，あるいは時間の関係で他の項目のなかで講義が行われているためと考えられる．講義時間に関しては大学間で顕著な差が認められた．歯科麻酔学の講義時間は各大学の教育方針や学部教育の時間をもとに決められていることも理由の一つと考えられる．歯科麻酔学教育と関係の深い医科系科目講義に関してはどの大学でも実施されていたが，総時間数や実施科目には顕著な差が認められた．医学部がある大学と単科大学で特にこのような差がみられていたが，大学に所属する医師数や併設診療科の数が影響していると考えられる．モデル・コア・カリキュラムでは歯科医療に必要な医学的知識（E-4-6）という項目があり，「歯科医師に必要な全身疾患（内科疾患）を理解する」という目標が定められている．最近の歯科医師国家試験では内科疾患など出題数が増加傾向にあるが，内科疾患の知識は歯科医療にとっても必須であり，今後も医科系科目の講義は増加していくと考えられる．

実習に関しても講義時間と同様に実習時間で大学間に大きな差が認められた．アンケートに示した実習項目はほとんどの大学で実施されていたが，実習形式には差が認められた．大学や診療科の方針，設備，教員数，臨床実習を受け入れる患者数などが大きく影響していると考えられる．臨床実習での歯科医療行為の条件として，侵襲性のそれほど高くない，一定のものに限られること，患者らの同意を得て実施することが示されることなどが挙げられている[7]．侵襲性のある項目では歯学教育モデル・コア・カリキュラムのなかで水準が示されており，歯科麻酔学実習に関連する項目も水準1から水準4まで示されている[8]．歯科麻酔学は専門性が高く，特に実習に関しては水準の高い項目もあり，学生の実習項目は制限されがちである．近年患者の権利の意識の高まり，医の倫理や生命倫理的な配慮の必要性から臨床の場のみにとどまらず臨床実習においてもインフォームドコンセントを行って同意を得るようになってきており，実習に影響を与えていると考えられる．今回の調査でも多くの大学ではモデル・コア・カリキュラムの水準に準拠して行っていたが，実習形式には差があった．水準3の静脈内鎮静法や水準4の全身麻酔に関しては見学のみで，気管挿

管はマネキンによる実習が行われていた．

浸潤麻酔に関しては，自験，相互実習，シミュレーション，見学など実習方式はさまざまであった．伝達麻酔は自験や相互自習を行っている大学は少なかったが，学生同士の相互実習における偶発症の問題があると考えられる．島田ら[9]の報告によると，下顎孔伝達麻酔実習において，局所麻酔実習を行っていたのは28校中25校で，伝達麻酔実習と浸潤麻酔実習の両方行っていたのは16校，下顎孔伝達麻酔実習のみは2校であったという．また，そのなかで全身偶発症は20例，局所的偶発症は16例発症しており，実習の同意を得ていたのは3校であったという．今回は実習に関して同意を得ているかは調査していないが，同意書をとっている大学は以前より増加していると思われる．今後の検討項目である．伝達麻酔はモデル・コア・カリキュラムでは水準2であるが，偶発症の発症を避けるためにシミュレーション実習を行っている大学も多いと考えられる．局所麻酔は歯科麻酔だけでなく口腔外科系実習，保存修復実習，クラウンブリッジ実習でも実施できることが行動目標で挙げられている[9]．そのため講義は歯科麻酔科で行われているのに実習は他科で実施されている大学もあり，講義と実習の連携の必要性があると考えられる．

一次救命処置とバイタルサインの測定は複数の学年で行っている大学が多かった．高石ら[10]は歯学部学生と卒直後歯科医師に対して行った救急蘇生教育に対する調査で学年が進むにつれ知識が低下していくことを報告している．共用試験対策としても行われていると考えられるが，これらの基本的知識と技術は歯科医師にとって重要であり，複数の学年で実施されていることは望ましい状況と考えられる．

注射法では静脈穿刺は相互実習とシミュレーション実習いずれも実施していない大学が約1/3あったが，静脈穿刺は緊急時の対応でも重要であり，静脈穿刺ができない場合は治療法の選択も限られる．水準2であることを考えるともう少し実施してもよいのかもしれない．過去に実態調査が30年前に行われているが[11]，今後検討が必要と考えられる．全身麻酔に関しては水準4であり，ほとんど見学実習であったのは当然の結果といえる．医学部においては全身麻酔シミュレーターなどを学生教育に活用している大学もあり有用性が報告されている[12]．しかし，高価であり普及は容易ではない．今回の調査において侵襲が大きい実習に関してはシミュレーション実習を行っている大学が多かった．文部科学省も一連の臨床の流れを一人の患者で経験することの必要性を重要視しているが，自験症例が十分に確保できない場合はシミュレーション実習を行うことを推奨しており，今後さらに増加していく可能性がある[5]．患者の全身管理にあたっては，一般的な管理法だけでなく有病者や高齢者の管理法を経験することは重要である．しかし，学生実習で配当される患者はさまざまであり，健康な患者も多く，有病者や高齢者の症例を全員が経験することは困難である．これを解決するために大学によっては麻酔計画立案などのシミュレーション実習を行って効果を上げている大学もある[13]．

モニタリングによる全身管理実習を行っていない大学が10校あったが，歯科麻酔科が担当せず担当診療科で行われる，あるいは実習時間の関係上省略されることが理由と考えられる．二次救命処置に関しても実習を行っているのは約半数で，実習を行っていてもその半数は座学実習であった．必須の目標でないため省略されることやシミュレーション機器が十分に設置されていないことなどが理由と考えられる．

歯科医療において歯科麻酔学は専門性が高く，卒後に歯科麻酔に関する教育を受ける機会は少ないため，歯科麻酔学の卒前教育はさらに重要性を増す．静脈内鎮静法に関しても学習や見学により有用性について理解が深まることで，将来の普及につながっていくことが期待できる．また，学生時代の医療安全に関する教育は卒後の医療安全の啓発にも有用である．

今回の調査では講義時間や実習時間，実習形式で大学間の差が大きかった．学生への講義や実習に関しては，文部科学省も大学それぞれの理念に基づく特色ある教育を推奨しており，講義や実習の時間数に差が生じるのは避けられない．しかし，歯科麻酔学の卒前教育は全身管理ができる有能な歯科医師の育成に必要不可欠である．講義と実習に関して時間数，項目，実習方法の基準を作成し，歯科麻酔学教育の標準化を図る，あるいはミニマムリクワイヤメントを作成する必要があると考えられた．

V．結　　語

現在，全国の歯学部・歯科大学で行われている歯科麻酔学の講義や実習の実態をある程度把握することができた．ほとんどの大学で必要な講義と実習が行われていたが，時間数や内容において大学間に差が認められた．今回の結果をもとに各大学で講義と実習の時間や内容について再検討する材料になれば幸いである．

大変ご多忙のなか，実態調査にご協力いただきました各大学の先生方に厚くお礼申し上げます．

本論文のすべての著者に開示すべき利益相反はない．

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Questionnaire Survey Regarding Dental Anesthesiology Education for Undergraduates

Undergraduate Education Working Group of the Japanese Dental Society of Anesthesiology
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and Junichiro KOTANI (previous member)

Abstract

An Undergraduate Education Working Group of the Japanese Dental Society of Anesthesiology conducted a questionnaire survey regarding dental anesthesiology education in dentistry departments and dental universities throughout Japan. After an initial trial survey of the 8 universities to which the committee members belonged, the questionnaire was revised and then distributed to the persons in charge of dental anesthesiology education at 29 universities throughout Japan between May and August, 2013. The questionnaire was divided into 2 sections regarding practical training (13 items, including length of training, year of students, and training method) and lectures (12 items, including number of lectures and year of students). Lectures on dental anesthesiology education were performed at all the universities, but the lecture times varied among the universities. Most of the lectures were delivered during the fourth year of university. Training in dental anesthesiology was also performed at all the universities, but the training times and the items and contents of the training varied widely among the universities. Most of the training sessions occurred during the fourth and fifth years of university. Simulation training was the most common practice method. The questionnaire results suggest that standardization of lecture times and contents as well as practices for both lectures and practical training should be considered.

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Clinical Paper
Orthognathic Surgery

Continuous postoperative pain control using a multiple-hole catheter after iliac bone grafting: comparison between ropivacaine and levobupivacaine

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Abstract. The aim of this study was to compare the analgesic effects of ropivacaine and levobupivacaine in continuous infiltration anaesthesia delivered via a multiple-hole catheter for the purpose of postoperative analgesia after iliac bone grafting. Thirty-four patients scheduled for iliac bone grafting in the maxillofacial region participated in this study. The patients were randomized to a ropivacaine group (Ropi group) and a levobupivacaine group (Levo group). After harvesting the iliac bone for grafting, a multiple-hole catheter was placed on the periosteum of the iliac bone. When surgery was completed, continuous administration was started at 4 ml/h of 0.2% ropivacaine (Ropi group) or 0.25% levobupivacaine (Levo group). Pain was evaluated in the recovery room and at 4 h after surgery, as well as at 9:00 and 18:00 on postoperative days 1, 2, and 3, using a visual analogue scale. Side effects were also recorded. No significant difference in the visual analogue scale scores at rest or in motion was observed between the two groups. In addition, there were no side effects in the two groups. Both 0.2% ropivacaine and 0.25% levobupivacaine provided comparable analgesic effects in continuous infiltration anaesthesia delivered via a multiple-hole catheter after iliac bone grafting.

Key words: postoperative pain; local anaesthesia; ropivacaine; levobupivacaine; iliac bone grafting.

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Iliac bone grafting is often performed for reconstruction of the jaw bone after tumour resection in the maxillofacial region. Postoperative pain related to the iliac crest wound can sometimes become worse than the intraoral pain, leading to patient distress and delays in postoperative ambulation.[1] Although the administration of opioid analgesics has been the conventional treatment for postoperative pain, respiratory depression and/or nausea and vomiting are clinical issues.[2] In recent years, postoperative analgesia using local anaesthetics or non-steroidal anti-inflammatory drugs has become common because these analgesics are not accompanied by the side-effects produced by opioids and therefore improve patient satisfaction.[3] It is reported that epidural anaesthesia is effective for the management of postoperative pain.[4] However, epidural anaesthesia requires expertise in the procedure and can lead to complications, including dural puncture, hypotension, and dysuria. Infiltration anaesthesia, which involves the continuous administration of local anaesthetics via an indwelling catheter placed in the iliac crest wound, is a safer procedure because there is no risk of these complications.[5–10] The use of multiple-hole catheters in the iliac crest wound for continuous postoperative pain control has been reported previously by this study group.[6]

The long-acting amide-type local anaesthetics ropivacaine and bupivacaine have been used in epidural anaesthesia and infiltration anaesthesia for many years. Ropivacaine has fewer cardiotoxic effects and shows earlier recovery of normal motor function than bupivacaine under similar sensory block.[11,12] Levobupivacaine is the pure *S*(−)-enantiomer of bupivacaine and is reported to be less cardiotoxic than racemic bupivacaine.[13] Levobupivacaine was developed for clinical use as a long-acting local anaesthetic after ropivacaine. Both ropivacaine and levobupivacaine are now commonly used for postoperative analgesia in various settings.[14–16]

Studies comparing ropivacaine and levobupivacaine in spinal anaesthesia and epidural anaesthesia have been published. In a study that compared both agents at the same concentration in epidural analgesia, the analgesic effects were reported to be comparable.[17] In contrast, in a study that calculated the minimum effective analgesic concentration (MEAC) for a comparison of both agents in epidural analgesia, levobupivacaine was 20% more potent than ropivacaine.[18] In addition, in other clinical studies comparing the two drugs in shoulder and knee surgery, similar analgesic effects were obtained at lower concentrations of levobupivacaine.[19,20] From these results, there is no consensus of opinion on the potency of these two agents. Therefore the present study was performed to compare the analgesic effects of ropivacaine and levobupivacaine in continuous infiltration anaesthesia delivered via a multiple-hole catheter for the purpose of postoperative analgesia after iliac bone grafting.

Methods

This research was performed with the approval of the dental college ethics committee. The study enrolled patients aged ≥18 years scheduled for iliac bone grafting in the maxillofacial region under general anaesthesia at the hospital between October 2011 and September 2013. These patients were classified as American Society of Anesthesiologists (ASA) physical status I or II, and all provided informed consent to participate. Patients were excluded if they had severe heart, liver, or kidney dysfunction, had a history of hypersensitivity to amide-type local anaesthetics, had a contraindication to the use of rescue analgesics (diclofenac sodium, loxoprofen sodium), had a haemostasis or blood coagulation abnormality, or were otherwise determined by the attending doctor to be unsuitable for study enrolment. The patients were randomized to either the ropivacaine group (Ropi group) or the levobupivacaine group (Levo group).

General anaesthesia was maintained either by inhalation anaesthesia using oxygen, air, and sevoflurane, or by total intravenous anaesthesia (TIVA) using oxygen, air, and propofol. Remifentanil or fentanyl was used for analgesia during surgery. The selection of the general anaesthetic and the dose of narcotic analgesic given during surgery were decided by the dental anaesthetists in charge.

To harvest the iliac bone for grafting, the oral surgeon administered 4 ml 1% lidocaine hydrochloride with 10 μg/ml epinephrine (Xylocaine Injection 1% with Epinephrine; AstraZeneca, Osaka, Japan) to the iliac bone harvest site by infiltration anaesthesia. After harvesting the iliac bone for grafting, a multiple-hole catheter (epidural catheter; Hakko, Tokyo, Japan) was placed on the periosteum of the iliac bone and the surgical incision in the skin was closed (Fig. 1). A 17-gauge, three-hole type multiple-hole catheter was used. The holes were located at 10, 30, and 50 mm from the catheter tip with each hole rotated by 180°. The catheter tip was semicircular in shape.

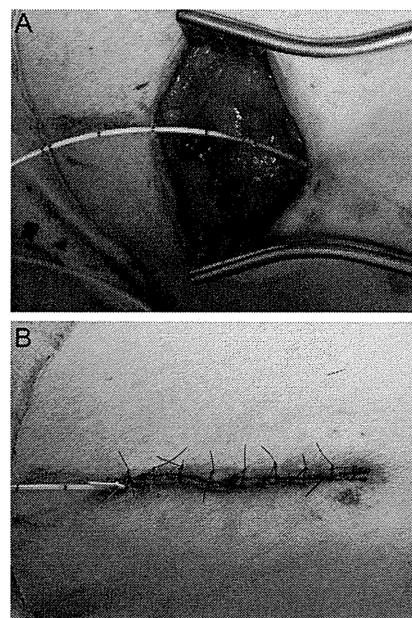

Fig. 1. A multiple-hole catheter placed in the iliac crest wound. (A) Before skin closure; the catheter is fixed on the periosteum. (B) After skin closure.

After suturing, the initial dose of infiltration anaesthesia was administered to the donor site: 5 ml 0.75% ropivacaine (Anapeine Injection; AstraZeneca, Osaka, Japan) in the Ropi group, or 5 ml 0.75% levobupivacaine (Popscaine 0.75% injection; Maruishi Pharmaceutical, Osaka, Japan) in the Levo group. When surgery to the maxillofacial region and iliac crest wound was completed, continuous administration was started at 4 ml/h of 0.2% ropivacaine in the Ropi group, or 0.25% levobupivacaine in the Levo group. The infusion was continued while the patient was transferred to the ward and for the next 48 h. After 48 h of infusion, an oral surgeon removed the catheter in the ward. A syringe-type disposable injector (Coopdech Syrinjector 120 ml, Ambulatory Infusion Pump; Daiken Medical, Osaka, Japan) was used for the continuous infiltration anaesthesia.

When the surgery was completed, all patients were administered diclofenac sodium 50 mg (Voltaren SUPPO; Novartis Pharmaceuticals, Tokyo, Japan) as a suppository. If the patient complained of pain in the ward and asked for rescue analgesics, diclofenac sodium (Voltaren Tablets; Novartis Pharmaceuticals, Tokyo, Japan) or loxoprofen sodium (Loxonin Tablets; Daiichi Sankyo, Tokyo, Japan) was administered orally. The maximum dose each time was 50 mg for diclofenac sodium

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and 120 mg for loxoprofen sodium, with doses given at least 4 h apart.

Pain at rest was evaluated in the recovery room after surgery and at 4 h after surgery, as well as at 09:00 and 18:00 on postoperative days 1, 2, and 3. Pain in motion was evaluated at 09:00 and 18:00 on postoperative days 1, 2, and 3. A visual analogue scale (VAS) ranging from 0 mm (no pain) to 100 mm (worst pain imaginable) was used. Pain at rest was defined as pain when resting in bed, whereas pain in motion was described as pain when the patient coughed, changed position, or was being transferred to a wheelchair. The start of walking without any support was decided by an oral surgeon. All pain evaluations were performed by one of the authors (H.K.). Data on the use of rescue analgesics in the ward, whether the patient experienced nausea and vomiting or dysuria, and when the patient resumed walking were also recorded.

Patient characteristics including age, height, weight, and body mass index (BMI) were analyzed using the Mann–Whitney U-test. This test was also used to analyze VAS scores. Fisher's exact test was used for other parameters. For all tests, a P-value of <0.05 was considered statistically significant.

Results

The study enrolled a total of 34 patients, with 17 patients in the Ropi group and 17 patients in the Levo group. The 17 patients in the Ropi group included three patients undergoing an iliac block bone graft and 14 patients undergoing a particulate cancellous bone and marrow (PCBM) graft. The 17 patients in the Levo group included four patients undergoing an iliac block bone graft and 13 patients undergoing a PCBM graft.

Table 1 shows the patient characteristics. No significant differences were observed between the two groups.

Table 2 shows the proportions (%) of patients complaining of pain at rest. For both groups, very few patients complained of pain when at rest after surgery. No significant differences were observed between the two groups.

Figure 2 shows the VAS scores for pain in motion. Although no significant differences were seen between the two groups, the VAS scores tended to be slightly lower in the Levo group than in the Ropi group on postoperative days 1 and 2.

Table 3 shows the results for the other items measured during the postoperative period. Three patients in the Ropi group and three patients in the Levo group requested rescue analgesics in the ward, with only one patient in each group complaining of pain related to the iliac crest wound; the other patients experienced pain in the maxillofacial region. The patient in the Ropi group who complained of pain from the iliac crest wound was administered loxoprofen sodium 60 mg on postoperative day 2, and the patient in the Levo group was administered diclofenac sodium 50 mg on postoperative day 1. Neither patient required further rescue analgesics. Of the two patients who complained of pain in the maxillofacial region in the Ropi group, one received loxoprofen sodium 120 mg and the other received diclofenac sodium 50 mg on postoperative day 2. Likewise, the two patients in the Levo group with maxillofacial region pain received diclofenac sodium 50 mg on postoperative day 2. All patients who complained of postoperative nausea and vomiting only experienced mild nausea on the day after surgery. The urinary catheter was removed the next morning in all patients. No dysuria or leg paralysis was observed thereafter. There

Table 1. Patient characteristics; mean ± SD.

	Ropivacaine group (n = 17)	Levobupivacaine group (n = 17)
Age, years	40.6 ± 15.8	41.0 ± 14.7
Gender, M/F	11/6	12/5
Height, cm	163.4 ± 9.1	166.9 ± 7.0
Weight, kg	61.3 ± 14.4	66.6 ± 17.2
BMI, kg/m^2	22.9 ± 4.3	23.8 ± 5.4
Duration of surgery, min	208 ± 113	185 ± 76
Anaesthesia		
Sevoflurane-based/TIVA	9/8	9/8
Fentanyl/fentanyl + remifentanil	12/5	10/7
Total dose of fentanyl, mg	0.40 ± 0.14	0.44 ± 0.14
Total dose of remifentanil, mg	3.50 ± 0.41	3.14 ± 1.97

SD, standard deviation; M, male; F, female; BMI, body mass index; TIVA, total intravenous anaesthesia.

Table 2. Proportion (%) of patients complaining of pain at rest.

	Ropivacaine group (n = 17), n (%)	Levobupivacaine group (n = 17), n (%)
Recovery room	0 (0)	1 (6)
4 h after surgery	0 (0)	1 (6)
Day 1 postoperative 9 a.m.	3 (18)	0 (0)
Day 1 postoperative 6 p.m.	3 (18)	1 (6)
Day 2 postoperative 9 a.m.	2 (12)	2 (12)
Day 2 postoperative 6 p.m.	3 (18)	1 (6)
Day 3 postoperative 9 a.m.	2 (12)	1 (6)
Day 3 postoperative 6 p.m.	2 (12)	1 (6)

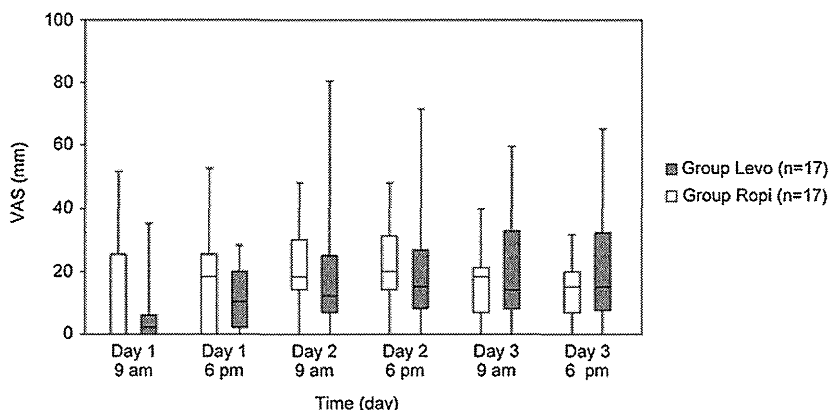

Fig. 2. Patient VAS scores for pain when in motion. The lines inside the boxes indicate the median values and the boxes indicate the 25–75% values. The ends of the vertical lines indicate the maximum and minimum values. White boxes: ropivacaine group (Ropi group); grey boxes: levobupivacaine group (Levo group). There were no statistically significant differences between the two groups.

Table 3. Other items measured during the postoperative period; mean ± SD.

	Ropivacaine group (n = 17)	Levobupivacaine group (n = 17)
Number of patients requiring rescue analgesics for iliac pain	1	1
Number of patients requiring rescue analgesics for oral pain	2	2
Postoperative nausea and vomiting	2	1
Duration of urinary catheter, h	16.4 ± 2.4	17.5 ± 7.6
Dysuria after removing the urinary catheter	0	0
Days required for the start of independent walking	4.1 ± 1.3	4.7 ± 2.0

SD, standard deviation.

were no serious cardiovascular side effects such as arrhythmia in either group, and no difference was observed between the two groups in terms of when the patients started independent walking. There were no other events of note.

Discussion

Seventeen patients in each group participated in this study. A preliminary study showed that the maximum VAS score in motion during continuous infiltration anaesthesia with ropivacaine was 40 ± 8 mm. A power analysis indicated that 17 patients were required to detect a stronger analgesic effect of levobupivacaine by 20% based on an α error of 0.05 and β error of 0.2. There was no control group receiving placebo in this study because it is extremely difficult to obtain approval from the ethics review board committee in Japan for a placebo controlled pain study.

As well as ropivacaine, levobupivacaine is commonly used as a long-acting anaesthetic agent in many countries. Each agent has been compared with other drugs in many clinical studies on postoperative pain control.

Pain from the iliac crest wound has been shown to be significantly lower during continuous infusion of 0.2% ropivacaine compared to normal saline.[5] Pain from the iliac crest wound has also been shown to be significantly lower during continuous infusion of 0.25% levobupivacaine compared to normal saline.[8] However, there has been no study comparing these two agents in postoperative analgesia after iliac bone grafting.

Namiki et al.[21] treated patients undergoing abdominal surgery with the continuous epidural administration of 0.2% ropivacaine or 0.25% levobupivacaine and did not observe any significant difference in the VAS scores at rest or in motion. However, they reported that the percentage of patients who did not require rescue analgesia during a 21-h period after surgery was significantly higher in the levobupivacaine group. The results from the present study also showed no significant difference in VAS scores for postoperative pain at rest or in motion, and the two agents produced almost equivalent analgesic effects.

The dissociation constant, liposolubility, and protein binding rate define the properties of a local anaesthetic; these factors can have a substantial effect on local anaesthetic action. Both ropivacaine and levobupivacaine have a dissociation constant of 8.2 and they have protein binding rates of 93.4 and 94, respectively. However, they have very different liposolubilities of 115 and 346, respectively.[22] Because more liposoluble local anaesthetics infiltrate into the tissues more readily and exert a more potent anaesthetic effect, these figures suggest that levobupivacaine may produce a more potent anaesthetic effect. McLeod and Columb[23] suggested that because ropivacaine has a molecular weight of 310.5 and levobupivacaine a molecular weight of 288, there are 7–8% more levobupivacaine molecules (24.2 mmol/l for ropivacaine and 26.0 mmol/l for levobupivacaine) in 0.75% solution, which might increase the nerve-block effect.

Animal studies also suggest that levobupivacaine produces a more potent anaesthetic effect than does ropivacaine.[24,25] Kanai et al.[24] inserted electrodes into the giant axons of crayfish and measured the action potential, maximum acceleration, and intracellular local anaesthetic concentration, and demonstrated that levobupivacaine produced a more potent nerve-blocking effect than did ropivacaine. A comparative study that used 0.25% ropivacaine and levobupivacaine on rat sciatic nerves suggested that nerve blockade generally lasted 30% longer with levobupivacaine.[25] Therefore, comparisons of these agents at the same concentration should show that levobupivacaine produces more potent effects.

The literature also includes studies in which ropivacaine and levobupivacaine have been compared in continuous infiltration anaesthesia, as in the present study. In a comparison of 0.2% ropivacaine and 0.125% levobupivacaine administered by continuous infiltration anaesthesia to patients undergoing open shoulder surgery, no significant difference in the VAS scores was observed. However, the total dose of patient-controlled interscalene analgesia (PCIA) within 24 h after surgery was lower in the patients administered levobupivacaine.[19] In a comparison of 0.2% ropivacaine and 0.2% or 0.125% levobupivacaine for sciatic nerve block administered by continuous infiltration anaesthesia in patients undergoing surgical correction of hallux valgus, no significant difference in the VAS scores at rest or in motion was seen between the groups.[26] These studies suggest that continuous infiltration anaesthesia produces good analgesic effects at lower concentrations with levobupivacaine than with ropivacaine.

Thus, both basic research and clinical studies suggest that levobupivacaine produces more potent anaesthetic and analgesic effects. In the present study, levobupivacaine was used at a concentration 0.05% higher than that of ropivacaine; hence, levobupivacaine should have shown a more potent analgesic effect. However, the results showed no significant difference. This might be because patients were not under significant load during bed rest, the iliac crest wound was small compared with that resulting from open shoulder surgery, and the patients experienced less pain. Furthermore, although no significant differences were observed on postoperative days 1 and 2, the VAS scores in the levobupivacaine group tended to be lower. This might indicate that the anaesthetic effect of levobupivacaine was more potent than that of ropivacaine.

Only one patient in each group used rescue analgesia for pain related to the iliac crest wound. This suggests that in both groups, continuous infiltration anaesthesia produced good analgesic effects and that both ropivacaine and levobupivacaine are useful agents for continuous infiltration anaesthesia after iliac bone grafting. Two patients in each group used rescue analgesia for pain in the maxillofacial region. This might have contributed to the alleviation of iliac pain.

Only a few patients in the two groups experienced mild postoperative nausea, with no difference observed between the groups. The two patients in the Ropi group who complained of postoperative nausea

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were given inhalation anaesthesia with sevoflurane, and the one patient in the Levo group was given anaesthesia by TIVA using remifentanil. It is suggested that the nausea in this study was caused by inhaled anaesthetic or opioid, because these symptoms disappeared the day after surgery.

In terms of circulatory side effects, research in animals and humans has not shown any clear difference in cardiotoxicity between ropivacaine and levobupivacaine.[27,28] Bleckner et al.[29] reported that the serum free ropivacaine concentrations under continuous infiltration anaesthesia remained well below toxic values despite relatively large amounts of ropivacaine (median ropivacaine dosage 21.6 mg/h (10.8 ml/h using 0.2% solution) for 7 days). This dosage was far larger than that used in the present study. Urinary catheters were removed in the morning of postoperative day 1 in nearly all patients and no change in urine volumes was observed. It is suggested that continuous infiltration anaesthesia can be performed safely without the risk of complications from hypotension or anuria seen with epidural anaesthesia.

No difference was seen between the two groups in terms of when the patients started independent walking. In a study by Morimoto et al.,[4] patients in the epidural group receiving continuous epidural anaesthesia started independent walking 4 days after iliac bone grafting, while patients in the control group started walking 8 to 9 days after grafting. In Japan, patients start independent walking on average more than 4 days after iliac bone grafting and the duration of the hospital stay is longer,[4] because the cost is fully covered by the national insurance system. The results of the present study suggest that continuous infusion anaesthesia has similar effects to continuous epidural anaesthesia.

In this study, the method of general anaesthesia and amounts of opioids used perioperatively could have affected the postoperative pain assessment. The general anaesthesia used in this study was either inhalation anaesthesia with sevoflurane or TIVA with propofol. Because patients awaken promptly after both types of general anaesthesia, it is suggested that there were no effects on postoperative pain the following day. Remifentanil used perioperatively is metabolized extremely rapidly, and fentanyl has a half-life of 3.6 h.[30] Therefore, these opioids might not affect the pain assessment at 4 h after surgery.

The donor sites in patients undergoing iliac PCBM grafting were almost the same size, at 5–7 cm long. In contrast, the donor sites in patients undergoing block grafting varied in size according to the size of the maxillofacial bone loss. However, because both groups included approximately the same number of PCBM and block graft patients, this difference would be expected to have a limited effect on the results. As all surgeons had clinical experience of more than 5 years and the same method was used for iliac bone harvesting, the difference in surgeons seems to have had little effect on the results. In this study, because the person recording the results was not blinded, this might have led to a bias. However, the results indicated no difference in analgesic effect between the two anaesthetics despite the anticipated superiority of levobupivacaine, suggesting that this bias might be minimal. The doses of ropivacaine and levobupivacaine were fixed for all patients in this study. Differences in their effects may have been observed with the use of patient-controlled analgesia (PCA) pumps, which allow the dose to be adjusted according to the patient's pain.

There are generally individual differences in pain levels even after the same surgery. Regarding the entry pain level, only one patient complained of pain immediately after surgery, with a VAS score of 12. This patient was in the Levo group; no patient in the Ropi group complained of pain immediately after surgery. However, this difference may not be a simple outcome of the difference in two local anaesthetics because there may have been residual effects of the fentanyl administered during surgery.

Despite including the number of patients indicated by the power analysis in this study ($n = 17$ per group), data from more patients and stratification of the pain level at onset may reveal differences in the analgesic effects between these two anaesthetics. Future investigations with more patients for the comparison of the two agents in concentration-based and/or pain level-based studies are recommended.

In conclusion, postoperative pain after iliac bone grafting was compared in patients administered 0.2% ropivacaine or 0.25% levobupivacaine using continuous infiltration anaesthesia via a multiple-hole catheter. The results showed no significant difference in the VAS scores in patients at rest or in motion. Because no serious side effects due to the drugs were observed, these results suggest that both agents can be used safely for postoperative analgesia. Continuous infiltration anaesthesia has fewer side-effects compared with epidural anaesthesia and opioid analgesics, and thus may accelerate the recovery to mobility after iliac bone grafting.

Funding

None.

Competing interests

None declared.

Ethical approval

This research was performed with the approval of the Tokyo Dental College Ethics Committee (Approval No. 302).

Patient consent

Not required.

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評価に特化した組織である．米国以外には韓国の Korean Institute of Dental Education and Evaluation (KIDEE) がある．

認証評価を行う場合には，評価側，受審側にとっても同一の枠組みが必要であり，認証評価基準 accreditation standards と呼ばれる．認証評価の方法はほぼ類似している．すなわち，認証評価基準に従って，各プログラムが自己評価報告書を作成し，その内容を立証する根拠資料とともに認証評価組織に提出する．自己評価報告書に基づいて，外部評価者による視察が行われ，視察報告書（案）が作成され，各プログラムからの反応があった後に，認証結果が確定されて，公表されるというものである．

認証結果が及ぼす影響については，英国の場合，国家試験がなく，GDC に歯科医師として登録されるためには，GDC により認証された歯科大学を卒業していることが必須である．仮にも認証が取り消された歯科大学が出た場合には影響は深刻である．米国の場合には歯科医師免許登録要件は各州政府が決めることになっているが，歯学教育，筆記試験，臨床試験の 3 要件があり，ほぼすべての州が歯学教育の要件として，CODA に認証されたプログラムを修了していることを挙げている．

以上のように歯科医学教育認証評価については，各国の歴史的背景や状況に応じて，それぞれの制度を発展・維持している．認証結果の影響力が大きいほど，それに割かれる時間やエネルギーは歯科大学側にとっても，認証評価実施組織にとっても大きなものとなる．

歯科医学教育認証評価トライアル修正版における評価項目・基準・観点・視点の紹介

一戸達也

近年，教育の質の保証が強く求められ，2004 年 4 月から国公私立のすべての高等教育機関は 7 年以内に 1 度，文部科学大臣の認証を受けた評価機関による評価（認証評価）を受審することが義務づけられた．認証評価には機関別認証評価と専門分野別認証評価とがあり，後者は法科大学院などで実施されている．医歯薬学系でも，社会に有為な人材を輩出するという，アウトカム基盤型教育の観点から後者がより重要になっており，薬学教育評価機構は 2013 年度から薬学教育の専門分野別認証評価を本格実施している．

歯学領域では，現在，文部科学省の補助金により，東京医科歯科大学・新潟大学・九州歯科大学・大阪歯科大学・東京歯科大学の 5 校が幹事校となって，2012 年度から 5 年計画で歯学教育の専門分野別認証評価のトライアルが行われている．昨年度は，九州歯科大学と大阪歯科大学がトライアルとしての専門分野別認証評価を受審し，歯学教育認証評価制度などの実施に関する調査研究 WG が立案した評価項目などによって自己点検評価書を作成して，それに基づいて実地調査を受けた．この経験と全国歯科大学・歯学部のご意見を踏まえて WG で評価項目などをブラッシュアップし，今年度は新潟大学と東京歯科大学がトライアルとしての認証評価を受審することになっている．

表 1　歯学教育認証評価項目（案）平成 27 年度版

1．教育の理念及び目標 (1)
2．学生の受け入れ (2)
3．歯学教育課程の内容・方法・環境 (4)
4．患者への配慮と臨床能力の確保 (2)
5．成績評価と卒業認定 (2)
6．教員組織 (2)
7．点検・評価 (2)

（ ）内の数は各項目に含まれる評価基準の数を示す．

評価項目は，表 1 に示す 7 項目からなっている．括弧内の数字は含まれる評価基準の数を示す．各評価基準には具体的な観点と視点が記載され，これらに基づいて自己点検評価書を作成することになる．評価項目のうち，2 はアドミッションポリシー，3 と 4 はカリキュラムポリシー，5 はディプロマポリシーを具現化した内容である．そして，歯学教育の特性から，カリキュラムポリシーのうち特に臨床実習に関連した部分を 4 として独立させている．なお，これらの評価項目などについては，「歯学教育認証制度等の実施に関する調査研究」のホームページ (http://www.cermed.jp/index.html) で公開されているので，参照していただきたい．

歯学教育の専門分野別認証評価とは，高いコンピテンシーをもった歯科医師を輩出するためのカリキュラムを大学が責任をもって構築し，そして実際にそのような人材が輩出されているのかどうかを，これらの評価項目に基づいて評価し，その教育カリキュラムを認証するということである．わが国の歯科医学教育が国民の目線でも国際的にも十分に信頼に足るものであることを保証するために，歯学教育認証評価制度のすみやかな確立が求められている．

認証評価トライアル（平成 26 年）実施状況について

北村知昭

現在，平成 24 年度文部科学省大学改革推進事業の一環として，基礎臨床を両輪とした医学教育改革によるグローバルな医師養成プログラムのなかで「歯学教育認証評価制度等の実施に関する調査研究」が 5 年間の取り組

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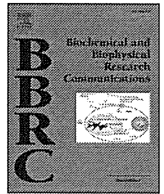

IL-33 inhibits RANKL-induced osteoclast formation through the regulation of Blimp-1 and IRF-8 expression

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ABSTRACT

Interleukin (IL)-33 is a recently discovered proinflammatory cytokine that belongs to the IL-1 family. Several studies have reported that IL-33 inhibits osteoclast differentiation. However, the mechanism of IL-33 regulation of osteoclastogenesis remains unclear. In the present study, we examined the effect of IL-33 on osteoclast formation *in vitro*. IL-33 suppressed osteoclast formation in both mouse bone marrow cells and monocyte/macrophage cell line RAW264.7 cells induced by receptor activator of NF-κB ligand (RANKL) and/or macrophage stimulating factor (M-CSF). IL-33 also inhibited the expression of RANKL-induced nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), thereby decreasing the expression of osteoclastogenesis-related marker genes, including *Cathepsin K*, *Osteoclast stimulatory transmembrane protein* (*Oc-stamp*) and *Tartrate-resistant acid phosphatase* (*Trap*). Blockage of IL-33-ST2 binding suppressed the IL-33-mediated inhibition of NFATc1. RANKL-induced B-lymphocyte-induced maturation protein-1 (Blimp-1) expression was also suppressed by IL-33, which was followed by the stimulation of anti-osteoclastic genes such as interferon regulatory factor-8 (IRF-8). These results suggest that IL-33-ST2 interactions down-regulate both RANKL-induced NFATc1 activation and osteoclast differentiation via the regulation of Blimp-1 and IRF-8 expression.

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1. Introduction

Bone remodeling is highly regulated by osteoblastic bone formation and osteoclastic bone resorption. These processes are strictly coupled in healthy bone by factors that include growth factors, hormones, and mechanical loading. Disorders of bone remodeling cause diseases such as rheumatoid arthritis, osteoporosis and osteopetrosis, as well as inflammatory bone resorption [1].

Osteoclast precursors interact with osteoblasts and stromal cells to permit their differentiation into mature osteoclasts [2]. Receptor activator of NF-κB ligand (RANKL) is expressed by osteoblasts/bone stromal cells. RANKL binding to the receptor RANK leads to the recruitment of intracellular tumor necrosis factor (TNF)-receptor-associated factor 6, and in turn, to the activation of the intercellular signaling pathway associated with nuclear factor of kappa B (NF-κB), mitogen-activated protein kinases, c-jun, and c-fos [3–5]. Finally, the interaction of RANKL with RANK induces the expression of the nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), the master regulator for osteoclastogenesis [6].

IL-33 is a recently discovered member of the IL-1 family of cytokines [7]. Full-length IL-33 is the bioactive form and it is released in the extracellular space in response to cell damage or mechanical injury. IL-33-mediated signaling involved its interaction with a heterodimeric receptor comprising ST2 and IL-1R accessory protein, leading to the activation of extracellular signal-regulated kinase 1/2 (Erk1/2), p38, c-jun N- terminal kinase (JNK), and NF-κB [8] and subsequently to the production of cytokines such as IL-1β,

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Interferon-γ (INF-γ), and TNF-α, which play a role in inflammatory diseases such as asthma, atopic dermatitis, rheumatoid arthritis, and anaphylactic shock [9].

IL-33 is expressed by bone-forming osteoblasts [10]. Moreover, recent studies reported that IL-33 was expressed in bone tissue, where it plays an important role in bone remodeling by effectively blocking osteoclastogenesis [10,11]; however, the mechanisms underlying the effects of IL-33 on osteoclast formation and function are largely unknown. In the present study, we used an *in vitro* culture system to investigate the molecular mechanisms by which IL-33 regulates osteoclastogenesis.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human IL-33 was obtained from R&D systems (Minneapolis, MN, USA). Recombinant human soluble RANKL and recombinant human macrophage colony stimulating factor (M-CSF) was purchased from Peprotech (Rocky Hill, NJ, USA). Anti-NFATc1 polyclonal antibodies and anti-Blimp-1 monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-IFN-regulatory-factor-8 (IRF-8), and anti-histone H3 monoclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

2.2. Cell culture

Bone marrow cells (BMCs) were isolated from femurs and tibias of 6-week-old male ddY mice (Kyudo Co., Ltd., Saga, Japan) and cultured in the presence of M-CSF (20 ng/mL) for 3 days. For differentiation into mature osteoclasts, osteoclast precursors (2.5×10^5 cells/well) were cultured with M-CSF (20 ng/mL) and RANKL (40 ng/mL) in the presence or absence of IL-33 on 24-well plate for 5 days. All procedures were approved by the Animal Care and Use Committee of Kyushu Dental University. The murine monocyte/macrophage cell line RAW264.7 was obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in α-minimal essential medium (α-MEM; Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS), penicillin G (100 units/mL), and streptomycin (100 ∝ g/mL). The cells were maintained at 37 °C in an atmosphere of 5% CO_2. Cells (1×10^3 cells/well) were cultured for 6 days with RANKL (40 ng/mL) in the presence or absence of IL-33 on 96-well plate to generate mature osteoclasts.

2.3. Evaluation of osteoclast differentiation

After culture, adherent cells were fixed and stained with tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma-Aldrich). TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclasts and were counted using a microscope.

2.4. Quantitative real-time RT-PCR

RAW264.7 cells (2×10^5 cells/well) were cultured for 48 h with RANKL (40 ng/mL) in the presence or absence of IL-33 (50 ng/mL). In some experiments, the cells were pre-treated with Rat IgG (37.5 μg/mL; R&D Systems) or with an anti-ST2 monoclonal antibody (37.5 μg/mL; R&D Systems) for 1 h prior to their stimulation with RANKL and IL-33. Total RNA was isolated from cells with an

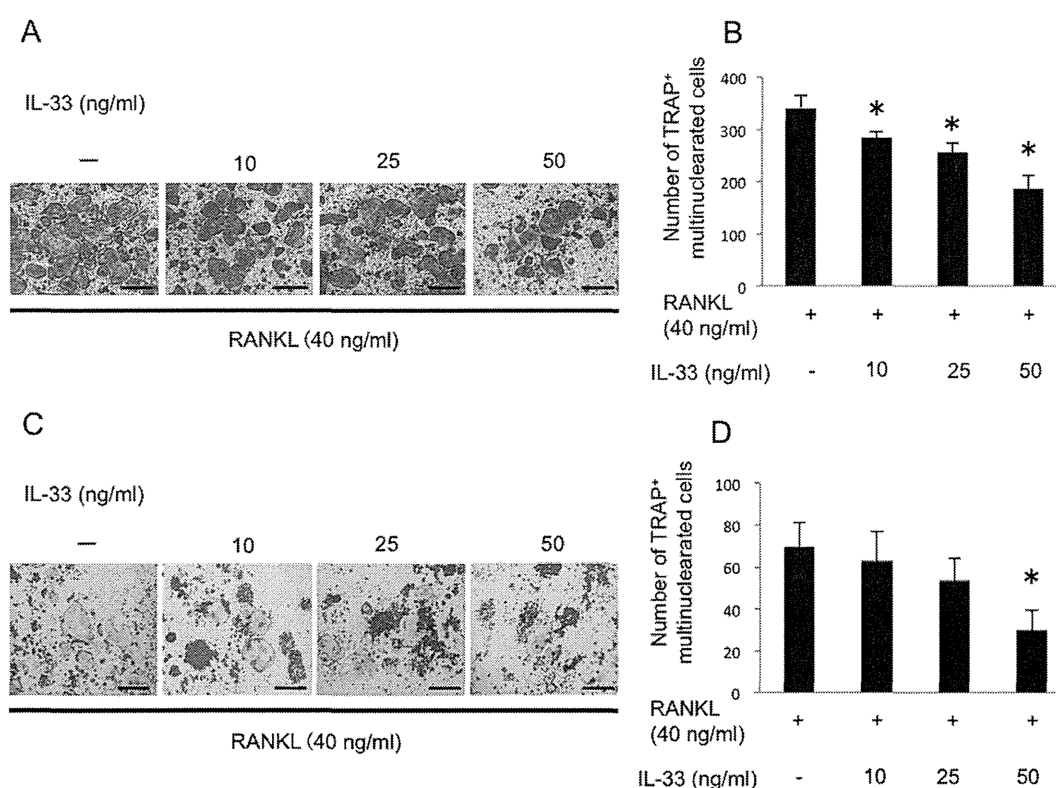

Fig. 1. Effect of IL-33 on osteoclast formation in BMCs and RAW264.7 cells. (A) BMCs were incubated with M-CSF and RANKL in the presence or absence of IL-33 for 5 days and then stained for TRAP activity. Scale bars show 500 μm. (B) Numbers of TRAP positive multinucleated cells were counted. (C) RAW264.7 cells were cultured with RANKL in the presence or absence of IL-33 for 6 days and then stained for TRAP activity. Scale bars show 500 μm. (D) Numbers of TRAP positive multinucleated cells were counted. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^*p < 0.05$ compared with the control without IL-33 treatment.

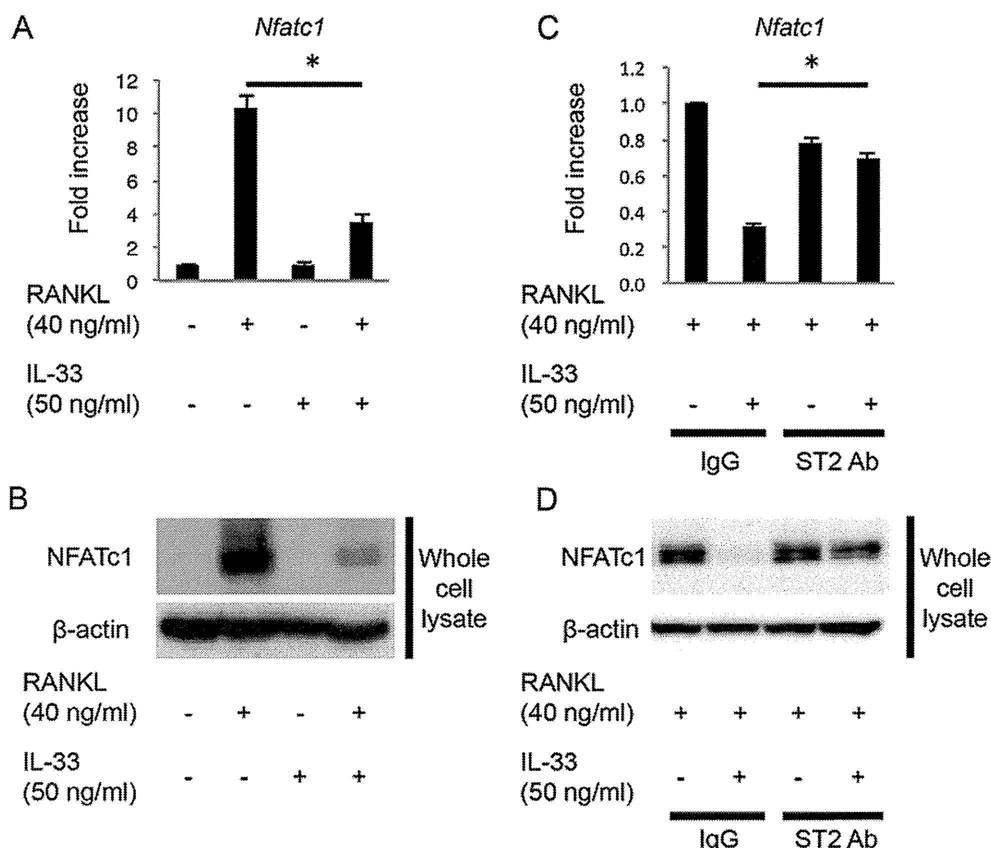

Fig. 2. Effect of IL-33 on RANKL-stimulated NFATc1 expression in RAW264.7 cells. RAW264.7 cells were stimulated with RANKL in the presence or absence of IL-33 for 48 h (real-time RT-PCR) or 72 h (Western Blotting). In neutralizing experiments, RAW264.7 cells were treated with a ST2 antibody for 1 h prior to stimulate with IL-33. (A), (C) The mRNA level of *Nfatc1* was measured by real-time RT-PCR. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^{*}p < 0.05$ compared with RANKL treatment. (B), (D) Whole cell lysates were subjected to SDS-PAGE and Western blot analyses, with the blots probed for NFATc1. Equivalent amounts of protein in the cell lysates were determined by measuring β-actin levels.

RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was transcribed with q-script cDNA supermix reagents (Quanta BioSciences, Gaithersburg, MD, USA). For real-time RT-PCR, the products were detected using the FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using the following primer sequences: *Gapdh*, 5′-GACGGCCGCATCTTCTTGA-3′ (forward) and 5′-CACACCGACCTT-CACCATTTT- 3′ (reverse); *Nfatc1*, 5′-ACCACCTTTCCGCAACCA-3′ (forward) and 5′-GGTACTGG- CTTCTCTTCCGTTTC-3′ (reverse); *Trap*, 5′-CTGCTGGGCCTACAAATCATA-3′ (forward) and 5′-GGGAGTCCT-CAGATCCATAGT-3′ (reverse); *Oc-stamp*, 5′-CCGCA- GCCTGA-CATTTGAG-3′ (forward) and 5′-TCTCCTGAGTGATCGTGTGCAT-3′ (reverse); *Cathepsin k*, 5′-TATGACCACTGCCTTCCAATAC- 3′ (forward) and 5′-GCC- GTGGCGTTATACATACA-3′ (reverse); *Blimp-1*, 5′-TTCTTGTGTGGTATTGTCGGG- ACTT-3′ (forward) and 5′-TTGGGGA-CACTCTTTGGGTAGAGTT-3′ (reverse); *Irf-8*, 5′-GGGCTGATCTGG-GAAAATGA-3′ (forward) and 5′-CACCTCCTGATTGTAATCC- TGCTT-3′ (reverse). Thermal cycling and fluorescence detection were performed using a StepOne real-time system (Applied Biosystems). Relative changes in gene expression were calculated using the comparative CT method. Total cDNA abundance between samples was normalized using primers specific to the GAPDH gene.

2.5. Western blot analysis

RAW264.7 cells (3×10^6 cells/well) were cultured with RANKL (40 ng/mL) in the presence or absence of IL-33 (50 ng/mL) for 48 or 72 h. In some experiments, the cells were pre-treated with Rat IgG (37.5 μg/m) or an anti-ST2 monoclonal antibody (37.5 μg/mL) for 1 h prior to their stimulation with RANKL and IL-33. Total protein was extracted using cell lysis buffer (Cell Signaling Technology Inc.) containing a protease inhibitor mixture (Thermo Fisher Scientific, Waltham, MA, USA) and a phosphatase inhibitor mixture (Nacalai Tesque Inc., Kyoto, Japan). Protein content was measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Non-specific binding sites were blocked for 30 min by immersing the membrane in Blocking One solution (Nacalai Tesque Inc.) at room temperature. The membranes were then incubated with diluted primary antibodies overnight 4 °C, followed by horseradish-peroxidase (HRP)-conjugated secondary antibodies for 60 min at room temperature. HRP-conjugated anti-mouse and anti-rabbit IgG (GE Healthcare, Little Chalfont, UK) or HRP-conjugated anti-rat IgG (Santa Cruz Biotechnology) antibodies were used as secondary antibodies. The membranes were washed and then treated with the ECL reagent (GE Healthcare) or Chemi-Lumi One Super (Nacalai Tesque Inc.). The resulting chemiluminescence detected digitally with GelDoc XR Plus (Bio-Rad Laboratories).

2.6. Nuclear translocation of NFATc1

RAW264.7 cells (3×10^6 cells/well) were cultured with RANKL (40 ng/mL) in the presence or absence of IL-33 (50 ng/mL) for 72 h. The cell pellets were treated with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manufacturer's instructions. Cell fractions were subjected to SDS-PAGE and immunoblotted with an antibody against NFATc1. In other

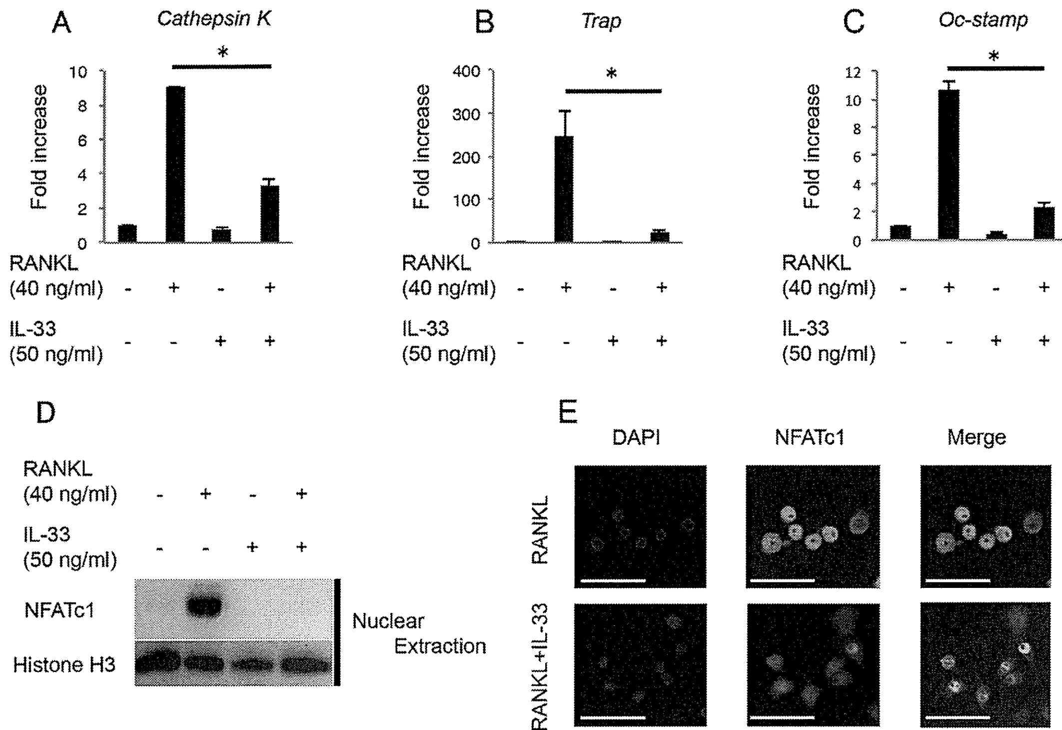

Fig. 3. Effect of IL-33 on the RANKL-induced expression of osteoclastogenic genes and NFATc1 translocation in RAW264.7 cells. The cells were stimulated with RANKL in the presence or absence of IL-33 for 48 h. The mRNA levels of *Cathepsin K* (A), *Trap* (B), and *Oc-stamp* (C) were measured by real-time RT-PCR. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^*p < 0.05$ compared with RANKL treatment. (D) RAW264.7 cells were stimulated with RANKL in the presence or absence of IL-33 for 72 h. Nuclear fractions were prepared and analyzed by Western blotting, probing the blots for NFATc1. Equivalent amounts of protein in the nuclear fractions were determined by measuring histone H3 levels. (E) RAW264.7 cells were stimulated with RANKL in the presence or absence of IL-33 for 12 h. The cells were fixed, permeabilized, and stained for NFATc1 (green) and nuclei (blue). Scale bars show 50 μm.

experiments, RAW264.7 cells (1 × 10^4 cells/well) were cultured with RANKL (40 ng/mL) in the presence or absence of IL-33 (50 ng/mL) for 12 h on 4-well chamber slides (Thermo Scientific) and then fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.2; PBS) for 60 min at 4 °C, followed by quenching with 0.2 M glycine in PBS. The cells were permeabilized using 0.2% Triton X-100 for 10 min at room temperature, blocked with 1% bovine serum albumin in PBS for 30 min, and then incubated with an anti-NFATc1 polyclonal antibody overnight at 4 °C. After a wash in PBS, the cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA), washed again, mounted in mounting medium containing 4′, 6-diamino-2-phenylidole (DAPI), and visualized using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). Images were captured digitally in real time and processed using BZ-II imaging software.

2.7. Statistical analysis

All data were obtained from three independent experiments, and each experiment was performed in triplicate. Statistical differences were determined using an unpaired Student's *t*-test with Bonferroni correction for multiple comparisons. All data are expressed as the mean ± standard deviation.

3. Results

3.1. IL-33 suppresses osteoclast formation in BMCs and RAW264.7 cells

To examine the effects of IL-33 on osteoclast differentiation, TRAP-positive multinucleated cells were counted as an indicator of osteoclast number. IL-33 inhibited the differentiation of BMCs into osteoclast-like cells mediated by M-CSF and RANKL. As shown in Fig. 1A and B, the effect of IL-33 was dose-dependent, with maximum inhibition observed at a concentration of 50 ng/mL (45.1 % inhibition). IL-33 also inhibited RANKL-induced differentiation of RAW264.7 cells into osteoclasts in a dose-dependent manner, with significant inhibition obtained at a concentration of 50 ng/mL (57.4% inhibition, Fig. 1C and D).

3.2. RANKL-stimulated expression of NFATc1 is inhibited by IL-33

In the intracellular signaling pathway of RANKL, NFATc1 is essential for osteoclast differentiation and is considered to be the master regulator for osteoclastogenesis. Therefore, we examined the effect of IL-33 on RANKL-induced NFATc1 expression by real-time RT-PCR and Western blotting. IL-33 significantly down-regulated the expression of RANKL-induced *Nfatc1* mRNA (Fig. 2A) and completely inhibited the expression of NFATc1 protein (Fig. 2B).

3.3. The IL-33 signaling pathway regulates NFATc1 expression via the ST2 receptor

To investigate the role of ST2 as an IL-33 receptor in the down-regulation of RANKL-induced NFATc1 expression, RAW264.7 cells were pre-treated with ST2 neutralizing antibody for 1 h prior to their stimulation with RANKL and IL-33. PCR and Western blot analysis showed that pre-treatment with the neutralizing antibody effectively prevented the IL-33-induced down-regulation of NFATc1 (Fig. 2C and D).

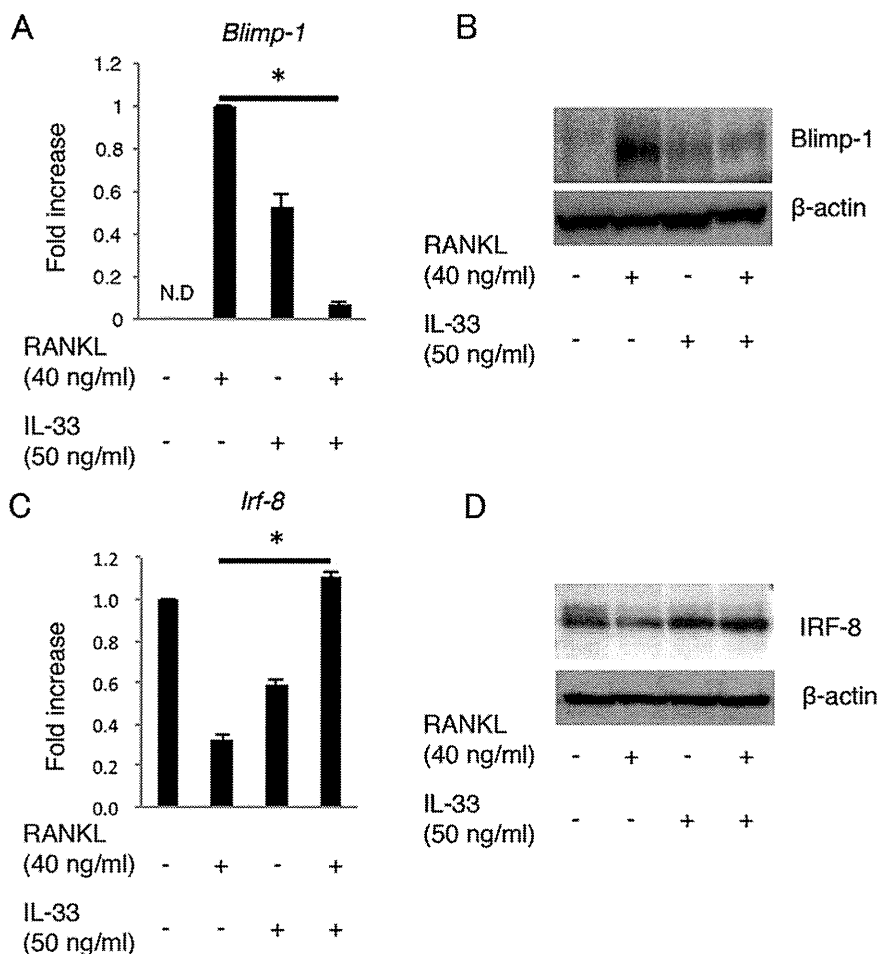

Fig. 4. Effect of IL-33 on Blimp-1 and IRF-8 expression in RAW264.7 cells induced by RANKL. The cells were stimulated with RANKL in the presence or absence of IL-33 for 48 h. The mRNA levels of *Blimp-1* (A) and *Irf-8* (C) were measured by real-time RT-PCR. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^{*}p < 0.05$ compared with RANKL treatment. Whole cell lysates were subjected to SDS-PAGE and Western blotting, with the blots probed for Blimp-1 (B) and IRF-8 (D). Equivalent amounts of protein in the cell lysates were determined by measuring β-actin levels.

3.4. IL-33 inhibits RANKL-induced osteoclast-related gene expression

The effect of IL-33 on osteoclast differentiation was investigated by measuring the expression levels of mRNAs encoding osteoclast-related genes in RAW264.7 cells after 48 h of RANKL stimulation. Consistent with the results of TRAP staining, IL-33 caused a significant decrease in the expression of osteoclast-related genes, such as *Cathepsin K* (Fig. 3A), *Trap* (Fig. 3B), *and Oc-stamp* (Fig. 3C) in RANKL-stimulated RAW264.7 cells.

3.5. IL-33 suppresses nuclear translocation of RANKL-induced NFATc1

NFATc1 is translocated from the cytoplasm to the nucleus by calcineurin-mediated dephosphorylation. Therefore, we investigated whether IL-33 altered RANKL-induced NFATc1 translocation. Nuclear translocation of NFATc1 was detected in RAW264.7 cells stimulated with RANKL. When the cells were incubated with both RANKL and IL-33, the level of NFATc1 protein in the nucleus was lower than that in cells treated with RANKL alone (Fig. 3D). Immunofluorescence analysis also revealed that nuclear translocation of NFATc1 in RAW264.7 cells induced by RANKL was decreased by IL-33 treatment (Fig. 3E).

3.6. IL-33 reduced Blimp-1 and recovered IRF-8 induced by RANKL at the gene expression level

We then analyzed the expression of Blimp-1, which is the transcriptional repressor of anti-osteoclastogenic genes and of IRF-8, the negative regulator of osteoclast differentiation. Our results showed that the RANKL-induced Blimp-1 expression was significantly suppressed by IL-33 (Fig. 4A and B). On the other hand, the down-regulation of IRF-8 induced by RANKL was recovered by the addition of IL-33 (Fig. 4C and D).

4. Discussion

Osteoclasts are large multinucleated cells formed by the fusion of precursor cells in the monocyte-macrophage lineage [12]. Their differentiation is dependent on growth factors, cytokines, and hormones. IL-33 is an alarmin cytokine and acts on a variety of cells, such as Th2 lymphocytes, mast cells, and macrophages [13,14]. Several studies reported that IL-33 was expressed by differentiated osteoblasts [15] and inhibited osteoclast formation [11,16,17].

Osteoclast formation of BMCs induced by M-CSF and RANKL was inhibited by the addition of IL-33 (Fig. 1A and B), indicating that IL-33 had a suppressive effect on osteoclastogenesis. We also found that IL-33 suppressed osteoclast formation in RAW264.7 cell

as well as BMCs when induced by RANKL (Fig. 1C and D). These findings suggest that IL-33 induces osteoclast differentiation through signaling pathways in the osteoclast precursor cells. NFATc1 is strongly induced by RANKL and is required for the terminal differentiation of osteoclasts [6,18]. The level of NFATc1 expression in RAW264.7 cells during osteoclastogenesis was suppressed by IL-33 (Fig. 2A and B), consistent with the down-regulation of osteoclast formation by IL-33 via the suppression of NFATc1.

Another important finding of our study is that the IL-33 receptor ST2 is required for the inhibition of RANKL-stimulated NFATc1 expression mediated by IL-33. The binding of IL-33 to ST2 is known to be involved in the onset of a variety of biological activities. A monoclonal anti-ST2 antibody is routinely used in IL-33-related blocking experiments [8]. As shown in Fig. 2C and D, pre-treatment with this antibody remarkably inhibited the effect of IL-33 on the down-regulation of NFATc1 in RAW264.7 cells. On the basis of these findings, we drew the connection of ST2 on RAW264.7 cells and IL-33 concerning the regulation of osteoclast formation and activation.

The inhibitory effect of IL-33 on osteoclastogenesis was confirmed by evaluating RANKL-induced expression of mRNAs of osteoclast-related genes. Cathepsin K [19] and TRAP [20] are related to the bone resorptive function of mature osteoclasts, and osteoclast stimulatory transmembrane protein (OC-STAMP) is associated with cell–cell fusion of osteoclasts [21]. It was demonstrated that *Cathepsin K*, *Trap* and *Oc-stamp* mRNA levels are regulated by NFATc1. Our results show that IL-33 notably decreased osteoclast-related gene expression regulated by *Nfatc1* in RAW264.7 cells (Fig. 3A, B and C). These results suggest that IL-33 modified the RANKL-NFATc1 signaling pathway, leading to a decrease in osteoclast formation.

During osteoclastogenesis, RANKL stimulation is found to induce the nuclear translocation of NFATc1. Furthermore, NFATc1 binds to its own promoter, which results in the robust induction of NFATc1. This autoamplification process of NAFTc1 is an important mechanism in osteoclastogenesis [22]. NFATc1 induction is also dependent on the activation of both the NF-κB [23] and the AP-1 complex containing c-fos [22]. However, IL-33 had no inhibitory effect on the NF-κB and AP-1 activation induced by RANKL (data not shown). So, we next confirmed the effect of IL-33 on NFATc1 nuclear translocation induced by RANKL and clarified that IL-33 interferes with RANKL-induced nuclear translocation of NFATc1 (Fig. 3D and E). Taken together, we conclude IL-33 suppresses RANKL-induced NFATc1 nuclear translocation through NF-κB- or AP-1-independent pathways during the differentiation of osteoclast precursors into mature osteoclasts. Further studies are needed to identify the other signaling molecules that are needed for osteoclastogenesis mediated by IL-33-ST2 interaction.

Recent studies reported that, during osteoclastogenesis, NFATc1 activity is negatively regulated by other transcription factors such as IRF-8, B cell lymphoma 6 (Bcl6), and v-Maf musculoaponeurotic fibrosarcoma oncogene family member protein B (MafB) [24–26]. Blimp-1 is a transcriptional repressor that plays crucial roles in the differentiation and/or function of numerous cell types, including macrophages and lymphocytes [27]. Furthermore, a genome wide screening of RANKL-inducible transcription factors revealed that Blimp-1 functions as a transcriptional repressor of *Irf-8*, *Bcl6*, and *Mafb* during osteoclastogenesis [24,25,28,29]. Interestingly, we found that the RANKL-induced Blimp-1 expression was down-regulated by IL-33 (Fig. 4A and B), while the suppression of IRF-8 expression mediated by RANKL was inhibited by IL-33 (Fig. 4C and D). IRF-8 is specifically expressed in immune cells, including macrophages [30]. IRF-8 binds to NFATc1 in osteoclast precursors and suppresses its transcriptional activity, resulting in the down-regulation of NFATc1 autoamplification and the expression of NFATc1 target osteoclast marker genes [24]. Taken together, these findings suggest that the inhibitory effect of IL-33 on RANKL-induced osteoclastogenesis involves modification of the Blimp-1-IRF-8 molecular axis.

In conclusion, we demonstrated that IL-33-ST2 interaction regulated Blimp-1 and IRF-8 expression and inhibited the activation of NFATc1 during RANKL-induced osteoclast formation. Although IL-33 strongly suppressed NFATc1 expression, it had only a moderate inhibitory effect on osteoclastogenesis induced by RANKL. Further elucidation of the molecular mechanisms of IL-33 in osteoclastogenesis will provide additional knowledge for homeostatic to prevent excessive bone resorption in both physiological and inflammatory conditions.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.033.

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ARTICLE

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Essential Role of Lysophosphatidylcholine Acyltransferase 3 in the Induction of Macrophage Polarization in PMA-Treated U937 Cells

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ABSTRACT

Lysophospholipid acyltransferases (LPLATs) regulate the diversification of fatty acid composition in biological membranes. Lysophosphatidylcholine acyltransferases (LPCATs) are members of the LPLATs that play a role in inflammatory responses. M1 macrophages differentiate in response to lipopolysaccharide (LPS) and are pro-inflammatory, whereas M2 macrophages, which differentiate in response to interleukin-4 (IL-4), are anti-inflammatory and involved in homeostasis and wound healing. In the present study, we showed that LPCATs play an important role in M1/M2-macrophage polarization. LPS changed the shape of PMA-treated U937 cells from rounded to spindle shaped and upregulated the mRNA and protein expression of the M1 macrophage markers CXCL10, TNF-α, and IL-1β. IL-4 had no effect on the shape of PMA-treated U937 cells and upregulated the M2 macrophage markers CD206, IL-1ra, and TGF-β in PMA-treated U937 cells. These results suggest that LPS and IL-4 promote the differentiation of PMA-treated U937 cells into M1- and M2-polarized macrophages, respectively. LPS significantly downregulated the mRNA expression of LPCAT3, one of four LPCAT isoforms, and suppressed its enzymatic activity toward linoleoyl-CoA and arachidonoyl-CoA in PMA-treated U937 cells. LPCAT3 knockdown induced a spindle-shaped morphology typical of M1-polarized macrophages, and increased the secretion of CXCL10 and decreased the levels of CD206 in IL-4-activated U937 cells. This indicates that knockdown of LPCAT3 shifts the differentiation of PMA-treated U937 cells to M1-polarized macrophages. Our findings suggest that LPCAT3

Abbreviations: BSA, bovine serum albumin; CXCL10, C-X-C motif ligand 10; DMSO, dimethyl sulfoxide; DPPC, dipalmitoyl-phosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FBS, fetal bovine serum; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-1ra, interleukin-1 receptor antagonist; IL-4, interleukin-4; LC-MS, liquid chromatography-mass spectrometry; LPC, lysophosphatidylcholine; LPCATs, lysophosphatidylcholine acyltransferases; LPLATs, lysophospholipid acyltransferases; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa-B; PAPC, palmitoyl-arachidonoyl-phosphatidylcholine; PC, phosphatidylcholine; PDPC, palmitoyl-docosahexaenoyl-phosphatidylcholine; PLPC, palmitoyl-linoleoyl-phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; POPC, palmitoyl-oleoyl-phosphatidylcholine; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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plays an important role in M1/M2-macrophage polarization, providing novel potential therapeutic targets for the regulation of immune and inflammatory disorders. J. Cell. Biochem. 116: 2840–2848, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: LYSOPHOSPHATIDYLCHOLINE ACYLTRANSFERASE 3; MACROPHAGE POLARIZATION; U937 CELLS

Glycerophospholipids are the major phospholipids in biological membranes and play an important role as the precursors of lipid mediators, including eicosanoids [Ishibashi et al., 2013], lysophospholipids [Kremer et al., 2010], and platelet-activating factor (PAF) [Snyder, 1989; Ishii and Shimizu, 2000] in the inflammatory response. Glycerophospholipids are synthesized from glycerol-3-phosphate in the de novo pathway (Kennedy pathway) [Kennedy and Weiss, 1956] and mature in the remodeling pathway called Lands' cycle [Lands, 1958]. In these processes, the recently identified lysophospholipid acyltransferases (LPLATs) play a major role in the configuration of the cellular membrane [Shindou et al., 2009; Harayama et al., 2014]. Through these pathways, phospholipids acquire diversity and asymmetry (*sn*-1 vs. *sn*-2) by reacylation and deacylation reactions catalyzed by LPLATs and phospholipases A_2s, respectively.

Lysophosphatidylcholine acyltransferases (LPCATs), which are members of LPLATs, are localized in the endoplasmic reticulum (ER) of many cell types and incorporate various fatty acids into the *sn*-2 position of lysophosphatidylcholine (LPC) to produce phosphatidylcholine (PC). Four LPCATs (LPCAT1-4) have been identified and functionally characterized to date [Shindou et al., 2009; Hishikawa et al., 2014]. Several studies have shown that LPCATs exert various biological functions. LPCAT1, which selectively incorporates palmitoyl-CoA into LPC as a substrate in addition to its involvement in pulmonary surfactant production [Nakanishi et al., 2006; Harayama et al., 2014], affects the progression of hepatocellular carcinoma [Morita et al., 2013]. LPCAT2 is activated by lipopolysaccharides (LPS) and produces the inflammatory lipid mediator PAF in mouse peritoneal macrophages [Shindou et al., 2007]. LPCAT3, which preferably incorporates linoleoyl-CoA and arachidonoyl-CoA into LPC [Hishikawa et al., 2008; Kazachkov et al., 2008; Zhao et al., 2008], upregulates the expression of peroxisome proliferator-activated receptor γ (PPARγ) and might be associated with adipocyte differentiation [Eto et al., 2012]. The liver X receptors (LXRs)-LPCAT3 pathway is an important modulator of inflammation [Rong et al., 2013]. LPCAT4 is involved in the deregulation of PC in colorectal cancer [Kurabe et al., 2013].

Macrophages are divided into at least two main classes known as M1 and M2 [Solinas et al., 2009]. When macrophages are exposed to LPS or interferon-γ (IFN-γ), they are polarized into M1 macrophages [Mosmann and Coffman, 1989], whereas exposure to interleukin-4 (IL-4) or IL-13 polarizes the cells into M2 macrophages [Abramson and Gallin, 1990]. M1-polarized macrophages produce pro-inflammatory chemokines and cytokines, such as C-X-C motif ligand 10 (CXCL10), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and infiltrate into injured tissue soon after damage [Arnold et al., 2007]. M2-polarized macrophages, whose markers are CD206, interleukin-1 receptor antagonist (IL-1ra), and transforming growth factor-β (TGF-β), are major resident macrophages and appear during the late stages of tissue repair and remodeling in injured tissue [Biswas and Mantovani, 2012].

Macrophages can switch from M1 to M2 or from M2 to M1 phenotypes according to their microenvironment or in response to certain stimuli [Gordon and Martinez, 2010; Biswas and Mantovani, 2012; Sica and Mantovani, 2012; Zhang et al., 2015]. Several mechanisms of M1/M2-macrophage polarization have been reported. The transcription factor nuclear factor-kappa-B (NF-κB) is a key player in M1/M2-macrophage polarization [Biswas and Lewis, 2010]. M2-polarized macrophages are epigenetically regulated by histone H3 lysine-4 and histone H3 lysine-27 methylation [Ishii et al., 2009]. PPARγ promotes the differentiation of human monocytes to M2-polarized macrophages [Bouhlel et al., 2007]. We speculated that M1/M2-macrophage polarization might be associated with LPCATs, as LPCATs and M1/M2-polarized macrophages play important roles in inflammatory responses. The relationship between LPCATs and M1/M2-macrophage polarization has not been reported to date.

In the present study, we investigated the physiological role of LPCAT3 in M1/M2-macrophage polarization and the underlying mechanism using human U937 cells. LPCAT3 mRNA expression was downregulated in LPS-activated U937 cells. Knockdown of LPCAT3 in PMA-treated U937 cells resulted in a shift to M1-polarized macrophages. Our results suggest that LPCAT3 exerts important anti-inflammatory effects mediated by the suppression of M1-macrophage polarization.

MATERIALS AND METHODS

REAGENTS

Recombinant human IL-4 was purchased from R&D Systems (Minneapolis, MN). LPS from *Escherichia coli* 0111:B4 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies and their respective sources were as follows: anti-IL-1β monoclonal antibody (Cell Signaling, Beverly, MA), anti-CD206 polyclonal antibody (R&D Systems), anti-IL-1ra monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX), anti-β-actin monoclonal antibody (Sigma–Aldrich), anti-goat IgG (Santa Cruz Biotechnology), anti-mouse IgG (GE Healthcare, Little Chalfont, UK). Deuterium-labeled 16:0 LPC, 16:0-, 18:1-, 18:2-, 20:4-, 22:6-CoA and dilauryl-PC were purchased from Avanti Polar Lipid (Alabaster, AL).

CELL CULTURE AND DIFFERENTIATION

Human monocytic leukemia U937 cells (RIKEN, RCB0435) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. U937 cells were seeded at a density of 5×10^5 cells/well into 6-well plates with RPMI 1640 containing 5% FBS and 100 ng/ml PMA. After 12 h of culture, the cells were washed with phosphate-buffered saline (PBS, pH 7.2)

and incubated with LPS (100 ng/ml) or IL-4 (20 ng/ml) for the indicated times.

MORPHOLOGICAL CHARACTERIZATION AND QUANTIFICATION

Images of cells were acquired by phase-contrast microscope (Olympus IX71, Tokyo, Japan). Cells were washed with PBS and further cultured in RPMI 1640 containing 5% FBS for 72 h. The quantification of morphological changes was performed based on the criteria used in the analysis of neurite outgrowth [Shea and Beermann, 1994]. Cells with more than twice the ratio of major axis to minor axis were defined as spindle-shaped cells. Spindle-shaped cells were counted in three randomly selected fields of triplicate cultures. Data are presented as a percentage of the total number of cells in the field.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA was extracted with an RNeasy® Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. cDNA was synthesized with q-Script™ cDNA SuperMix reagents (Quanta BioSciences, Gaitherburg, MD). qRT-PCR analysis was performed by StepOne™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA). The reaction for each gene was performed using Fast SYBR® Green Master Mix (Applied Biosystems). Relative quantification was calculated as a ratio of gene expression to the reference housekeeping gene, 18S rRNA. The sequences of the primer pairs used in this analysis are presented in Table I.

PHAGOCYTOSIS ASSAY

Macrophage phagocytic activity was quantified using a Phagocytosis Assay Kit, IgG FITC (Cayman Chemical, Ann Arbor, MI). Briefly, cells were seeded into 6-well plates at a density of 5×10^5 cells/well and treated with PMA (100 ng/ml) for 12 h. The cells were washed twice with PBS and stimulated with LPS or IL-4. After 48 h of culture, 100 μl of the latex beads-rabbit IgG-FITC solution was added to each well. The cells were incubated for 24 h and then washed twice with PBS to remove the latex beads. All cells were analyzed using an EPICS XL (Beckman Coulter, Fullerton, CA). Flow cytometric measurement was performed in the FL-1 channel.

FLOW CYTOMETRY

PMA-treated U937 cells (5×10^5 cells) were cultured with IL-4 (20 ng/ml) for 72 h. After washing with PBS supplemented with 1% bovine serum albumin (BSA) and 0.1% NaN_3, cells were resuspended in PBS. Cells were then incubated in Clear Back (human Fc receptor blocking reagent; MBL, Nagoya, Japan) for 10 min followed by incubation with FITC-Mouse Anti-Human CD206 (BD Biosciences) for 30 min at room temperature in the dark. After the final washing step, labeled cells were analyzed by flow cytometry.

WESTERN BLOT ANALYSIS

Cells were lysed in sodium dodecyl sulfate (SDS) buffer (50 mM Tris–HCl, 2% SDS; pH 6.8), and the protein concentration of the lysate was measured using a DC-protein assay kit (Bio-Rad, Hercules, CA). Lysates were boiled at 95°C for 5 min in SDS loading buffer. Electrophoretic separation (10–25 μg protein/lane) was carried out on 7.5–15% polyacrylamide gels and then proteins transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked for 30 min at room temperature with Blocking One (NACALAI TESQUE, Inc., Kyoto, Japan) and incubated overnight at 4°C with 0.1% Tween-20 in PBS (PBS-T) containing the primary antibody. After washing with PBS-T, the membranes were coated with secondary antibody in PBS for 1 h at room temperature. After washing the membranes with PBS-T, chemiluminescence detection was performed using ECL reagent (Amersham Pharmacia Biotech, Uppsala, Sweden). Results were visualized using a Molecular Imager® ChemiDoc™ XRS Plus system (Bio-Rad). Densitometric analysis of protein bands was performed using Image Lab® (Bio-Rad, Munich, Germany). Relative expression was calculated by dividing the band intensities of the proteins of interest by that of β-actin.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

PMA-treated U937 cells (5×10^5 cells) were cultured with or without LPS (100 ng/ml) or IL-4 (20 ng/ml). The supernatants from stimulated U937 cells were collected at 72 h. The concentrations of CXCL10, TNF-α, and TGF-β in the culture supernatants were determined using an ELISA kit (R&D systems) according to the manufacturer's instructions.

PROTEIN PREPARATION AND MEASUREMENT OF LPCAT ACTIVITY

U937 cells were scraped into 1 ml of ice-cold buffer containing 20 mM Tris–HCl (pH 7.4), 300 mM sucrose, and proteinase inhibitor cocktail (Complete, Roche, Basel, Switzerland). The cells were sonicated on ice three times for 30 s using a probe sonicator (Ohtake Works, Tokyo, Japan). After centrifugation at 9,000*g* for 10 min, the supernatants were centrifuged at 100,000*g* for 1 h. Resultant pellets were suspended

TABLE I. The Sequences of the Primer Pairs Used in This Analysis Are Indicated

Name	Sense primer, 5′→3′	Antisense primer, 5′→3′
18S rRNA	CGAACGTCTGCCCTATCAACTT	ACCCGTGGTCACCATGGTA
CD68	AGGCTGTGGGTGGGATCA	CTTGGAAAGGAGGAAATGAAAGTC
CXCL10	TTCCTGCAAGCCAATTTTGTC	TCTTCTCACCCTTCTTTTTCATTGT
TNF-α	GCAGGTCTACTTTGGGATCATTG	GCGTTTGGGAAGGTTGGA
IL-1β	TCAGCCAATCTTCATTGCTCAA	TGGCGAGCTCAGGTACTTCTG
CD206	CGCTACTAGGCAATGCCAATG	GCAATCTGCGTACCACTTGTTTT
IL-1ra	CTGCACAGCGATGGAAGCT	GCCTTCGTCAGGCATATTGG
TGF-β	CGCGTGCTAATGGTGGAAA	GCTGTGTGTACTCTGCTTGAACTTGT
LPCAT1	TTGCTTCCAATTCGTGTCTTATT	ATCCCATTGAAAAGAACATAGCA
LPCAT2	CCTCATGACACTGACGCTCTTC	CAGGAAGTCCACAACCTTCCTC
LPCAT3	CATTGCCTCATTCAACATCAACA	AGGAATTCCATCTGGAAGCAGAC
LPCAT4	AAGGTGGCGTTGGAACCA	CCCAGCCTTCCGAAGCA