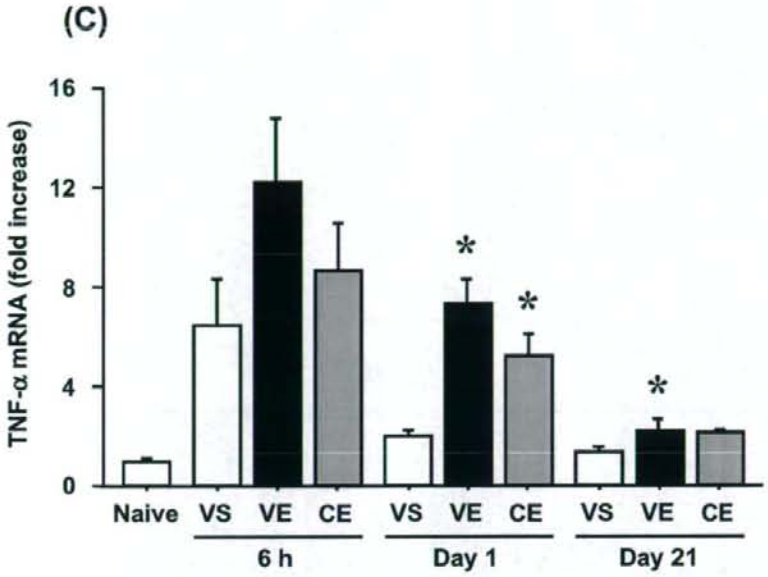

(C)
TNF-α mRNA (fold increase)
16
12
8
4
0
*
*
*
Naive
VS
VE
CE
VS
VE
CE
VS
VE
CE
6 h
Day 1
Day 21

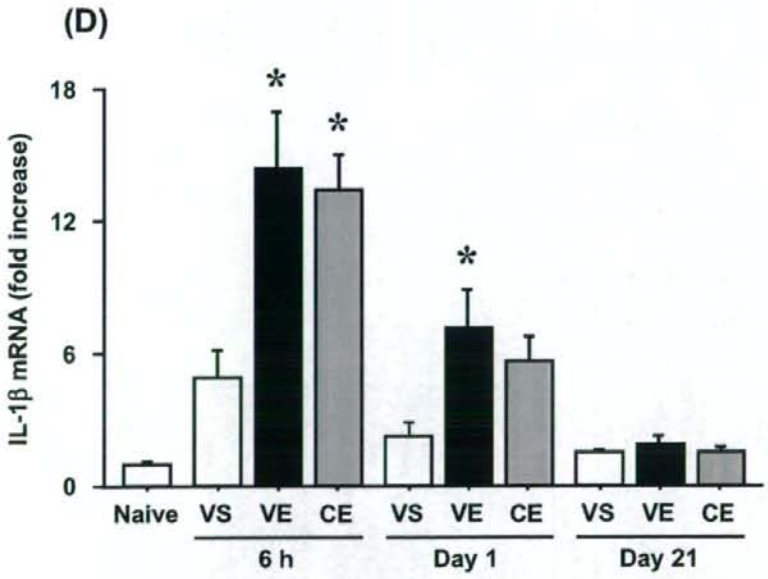

(D)
IL-1β mRNA (fold increase)
18
12
6
0
*
*
*
Naive
VS
VE
CE
VS
VE
CE
VS
VE
CE
6 h
Day 1
Day 21

Figure 6

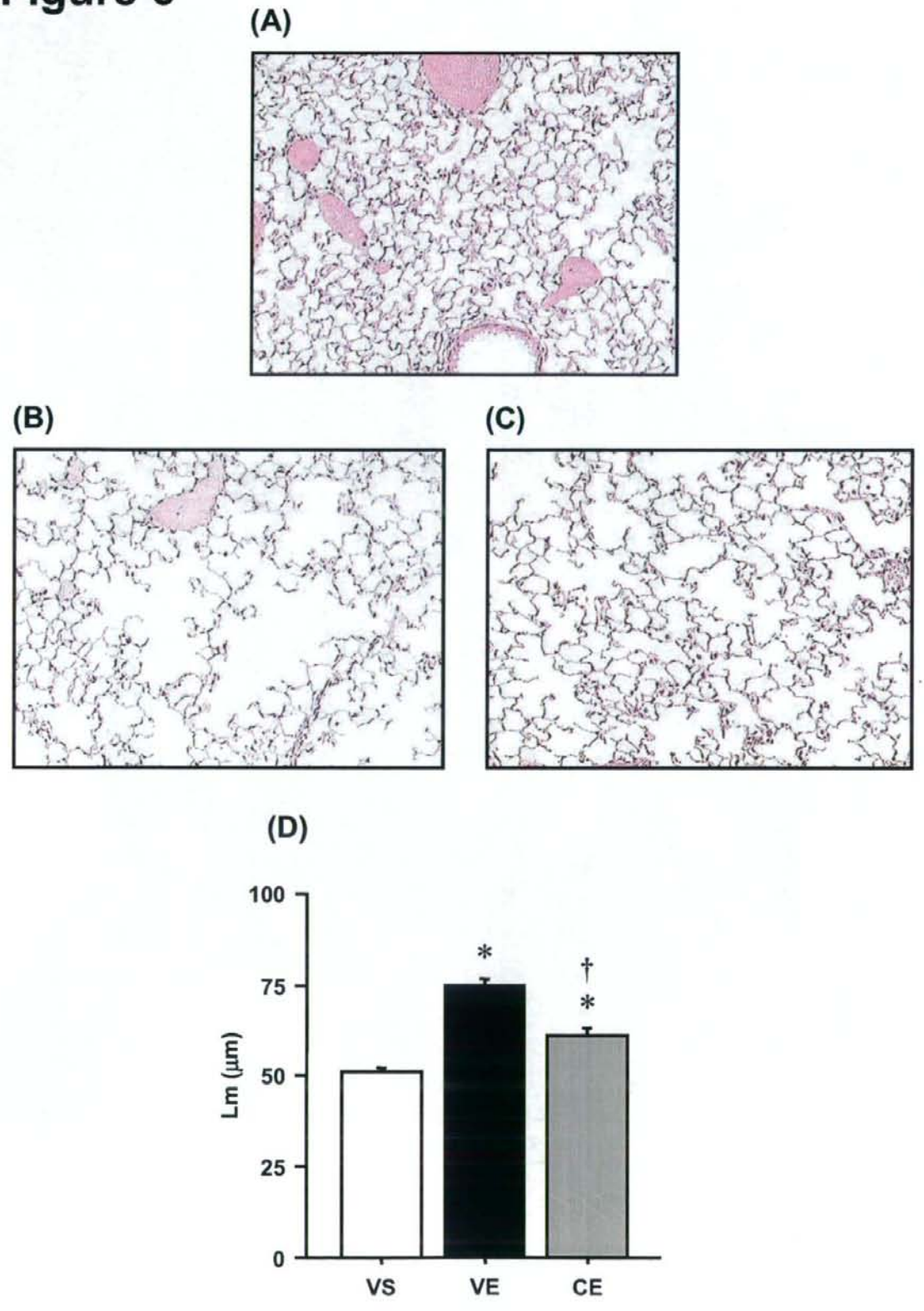


Figure 7

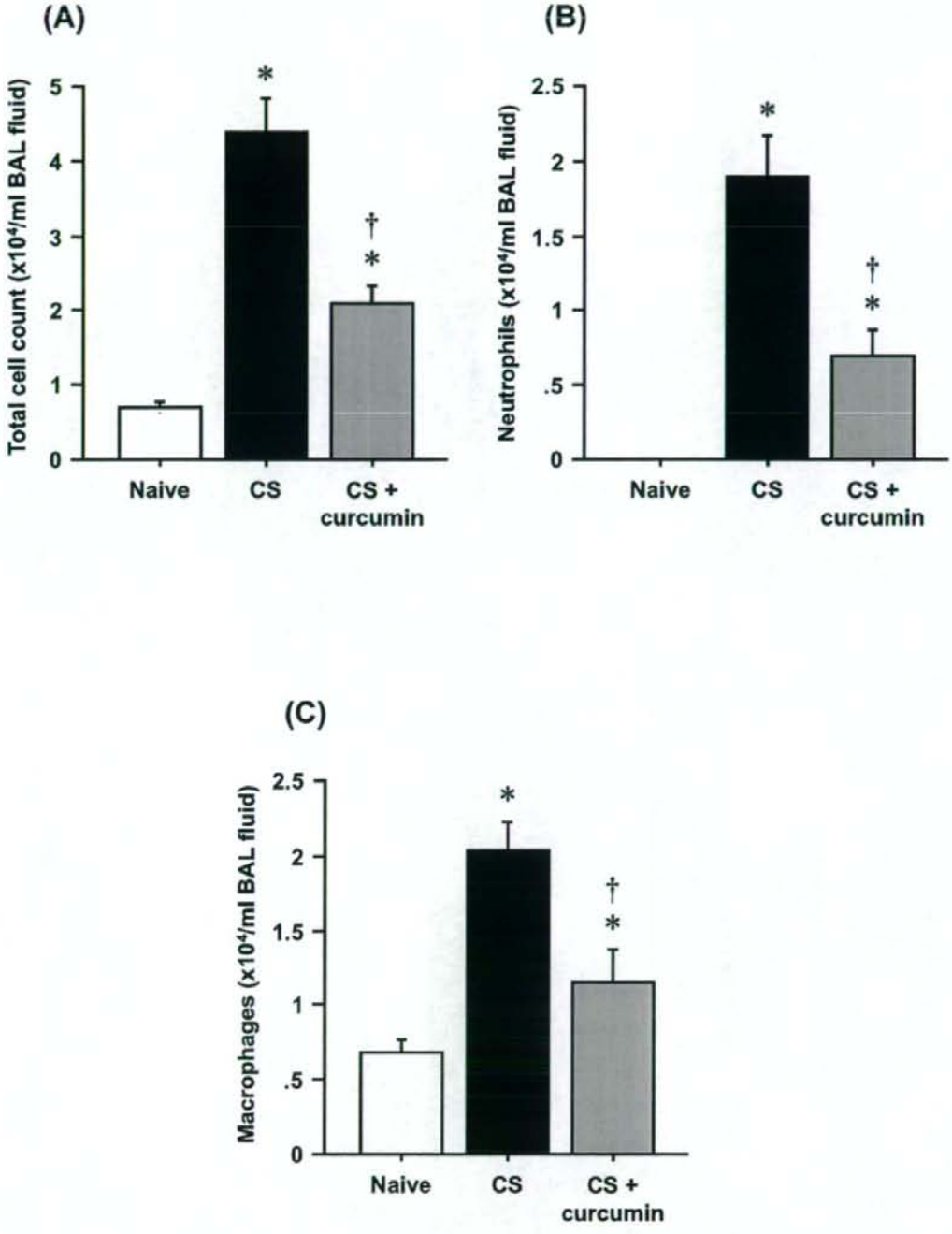

(A)
Total cell count (x10⁴/ml BAL fluid)
5
4
3
2
1
0
*
†
*
Naive
CS
CS + curcumin
(B)
Neutrophils (x10⁴/ml BAL fluid)
2.5
2
1.5
1
.5
0
*
†
*
Naive
CS
CS + curcumin
(C)
Macrophages (x10⁴/ml BAL fluid)
2.5
2
1.5
1
.5
0
*
†
*
Naive
CS
CS + curcumin

Figure 8

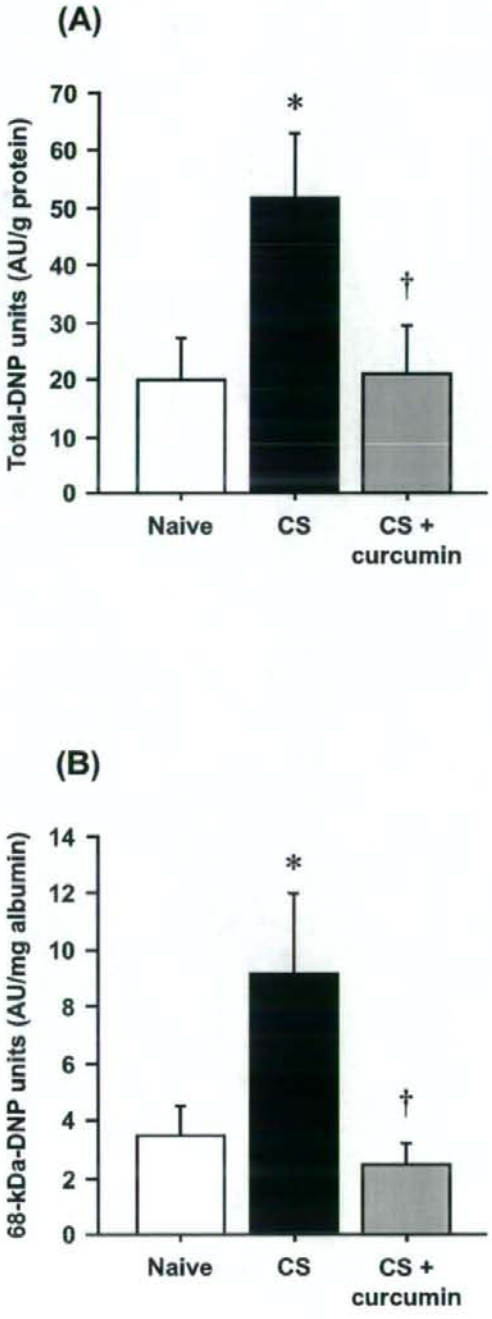
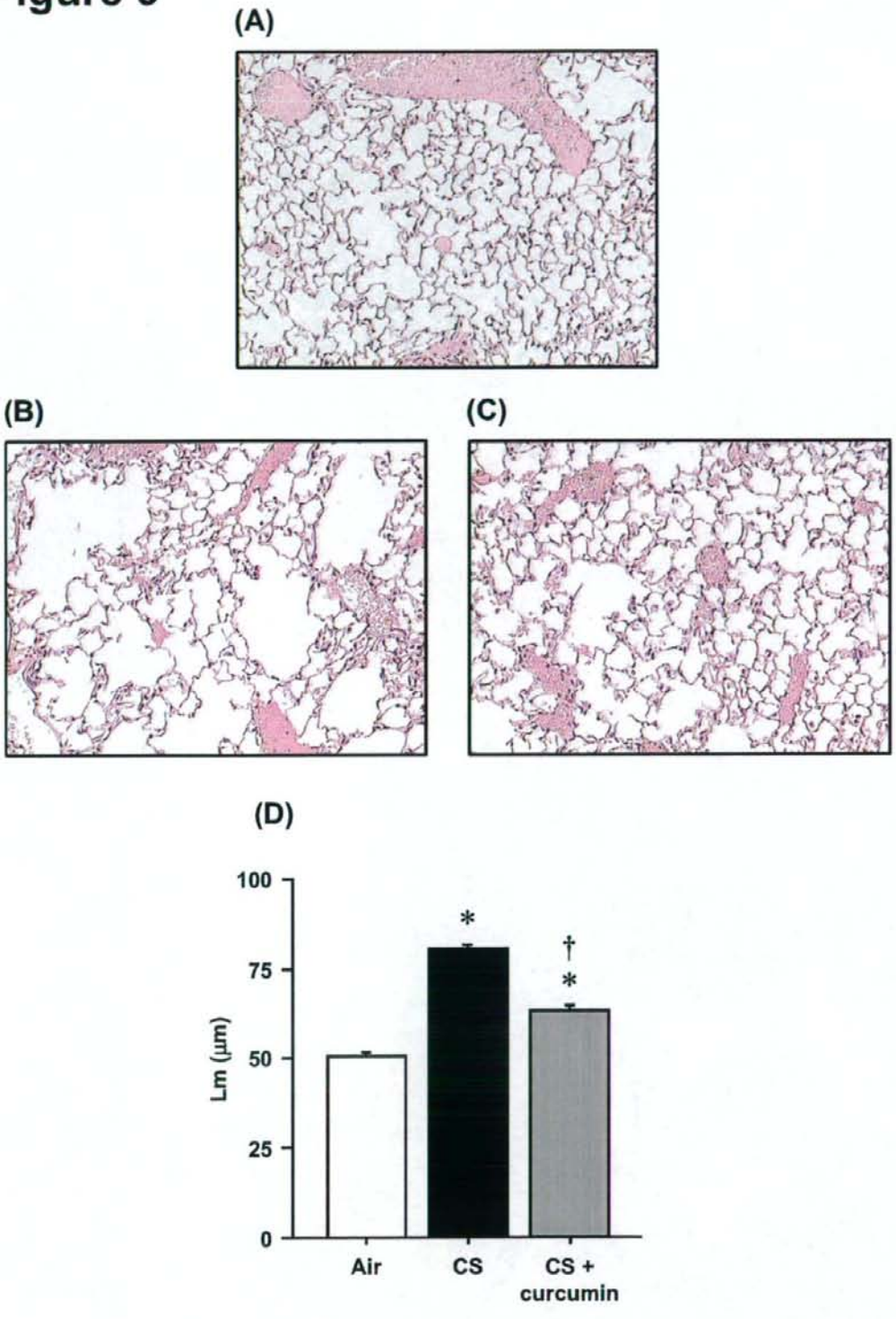

(A)
Total-DNP units (AU/g protein)
70
60
50
40
30
20
10
0
*
†
Naive
CS
CS + curcumin
(B)
68-kDa-DNP units (AU/mg albumin)
14
12
10
8
6
4
2
0
*
†
Naive
CS
CS + curcumin

Figure 9



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KNEE

A soluble factor (EMMPRIN) in exudate influences knee motion after total arthroplasty

Jun Onodera · Shin Onodera · Eiji Kondo · Tomoko Betsuyaku · Kazunori Yasuda

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Abstract Few studies have been conducted to investigate biological factors that affect postoperative knee motion after total knee arthroplasty (TKA). The purpose of this study is to test the hypothesis that range of knee motion (ROM) at 4 weeks after TKA is correlated with the concentration of extracellular matrix metalloproteinase inducer (EMMPRIN) and transforming growth factor (TGF)-beta1 in the exudative fluid harvested from the joint after surgery. A prospective measurement study was conducted with 20 osteoarthritis patients who underwent TKA. At 48 h after surgery, the exudate was harvested from a closed drainage system. Enzyme-linked immunosorbent assay was performed to measure the concentration of TGF-beta1, EMMPRIN, MMP-1, 2, 9, tissue inhibitor of metalloproteinase-1, and Hyalunonan. Knee flexion angle was measured before and at 4 weeks after surgery. There was a significant correlation between the EMMPRIN levels and knee flexion angle ($r = 0.557$, $p = 0.0148$). Western blot analysis of the exudate showed a prominent band for EMMPRIN at 27 kDa. On the other hand, there was no correlation between the TGF-beta1 levels and the knee flexion angle. This study showed that EMMPRIN levels after TKA affect the postoperative ROM. As to clinical relevance, EMMPRIN in the exudate after TKA is a promising biological indicator to predict difficulty in restoring postoperative ROM.

Keywords Total knee arthroplasty · Range of knee motion · Fibrosis · Transforming growth factor · EMMPRIN · MMP

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Introduction

Total knee arthroplasty (TKA) is an effective surgical treatment for patients suffering from serious joint destruction. However, one of serious problems after TKA is frequent occurrence of remarkable reduction in range of motion (ROM). Previous studies have reported that causes of the postoperative ROM reduction include preoperative loss of ROM [12, 23, 33], inappropriate prosthesis design [7, 34], failure in ligament balancing during surgery [30], and insufficient post-operative rehabilitation [2, 21]. Clinically, orthopedic surgeons have speculated that fibrosis of the knee joint capsule is a common cause of ROM reduction after TKA, because there are many patients who suffer from the so-called "stiff" knee in spite of excellent surgery with a theoretically flexible prosthesis and great effort in the post-operative rehabilitation. Fibrosis is a biological phenomenon that results from disruption in the homeostasis between the collagen anabolism and catabolism. Therefore, reduction in the ROM after TKA should be studied not only on the mechanical and therapeutic factors but also on biological factors affecting collagen metabolism in the tissues surrounding the knee joint. However, few studies have been conducted to investigate the biological factors affecting postoperative knee flexion angle after TKA.

We hypothesize that the postoperative ROM, specifically the range at the early phase after TKA, may be

significantly affected by the collagen metabolism in each patient. The collagen metabolism is controlled by a number of soluble factors. Previous studies reported that the synovial fluid contains various soluble factors that affect anabolic and catabolic mechanisms of collagen and proteoglycan accumulation [9, 24, 40]. Therefore, in the present study, we investigate exudative fluid from the knee joint, which can be harvested from a continuous drainage system post surgery, because this fluid contains various soluble factors that may affect the collagen metabolism in the tissues surrounding the knee joint. In the exudative fluid, we have focused on extracellular matrix metalloproteinase inducer (EMMPRIN) and transforming growth factor (TGF)-beta1 for the following reasons.

EMMPRIN, also known as CD147, basigin, or OX47, is a glycosylated transmembrane protein that has been identified as a member of the immunoglobulin superfamily [4]. One defining property of EMMPRIN is its capacity to stimulate production of various matrix metalloproteinases (MMPs) [43], and EMMPRIN has recently been getting a lot more attention because it is up-regulated in diverse pathologic processes of various organs in association with various MMPs [3, 6, 11, 31, 39]. Although it is essentially absent in normal synovial fluid, upregulation has been reported in rheumatoid arthritis [20, 42]. On the other hand, TGF-beta1 is also known to play an important role in the regulation of extracellular matrix protein production [28]. Previous studies have already reported that over-expression of TGF-beta1 in the joint capsule induces contracture of the joint [15, 32, 36].

Thus, the authors have hypothesized that the ROM at 4 weeks after TKA is correlated with the concentration of EMMPRIN and TGF-beta1 in the exudate harvested from the joint at 48 h after surgery. The purpose of this study is to test this hypothesis. Additional aims of this study are to analyze the relationship between the EMMPRIN or TGF-beta1 concentration and the concentration of MMPs, tissue inhibitor of metalloproteinase (TIMP)-1, and hyarunonan (HA), in order to clarify the mechanism of the effect of EMMPRIN or TGF-beta1 on the ROM at 4 weeks after TKA.

Materials and methods

Study design

A prospective measurement study was conducted with 20 patients who underwent unilateral TKA due to severe medial osteoarthritis in our hospital, under the Role and Regulation of the Human Research Committee, Hokkaido University. A TKA was indicated for severe disability resulting from pain, deformity, and limited function. Surgery was considered only after an adequate period of conservative therapy failed, which included physical therapy, anti-inflammatory medication, and modification of daily activities. The contraindications included inactive or latent infection, lack of muscle control, and inability to cooperate in the postoperative period. Preoperative exclusion criteria for this study included varus knee deformity of more than 16°, extension loss of more than 10°, range of knee motion (ROM) of less than 90°, and instability of more than 10°. Intra-operative exclusion criteria involved a knee that failed to obtain full extension or flexion of more than 140° in passive motion during surgery or a knee that had remarkable instability.

Patients were preoperatively informed about the below-described measurement protocol. One experienced senior orthopedic surgeon (K. Y.) performed all TKA operations using the posterior cruciate ligament-retaining type components of the LFA-III total knee (Kyocera, Kyoto, Japan), which has been commercially available with excellent clinical results in Japan since 1999 [27, 44, 45]. The femoral component of the LFA-III total knee was made of Zirconia ceramics. The tibial component consisted of a UHMWPE insert and a titanium tibial tray with a square-shaped stem. During surgery, a drainage tube was inserted into the knee joint and connected to a closed continuous drainage system (SB-Vac®, Sumitomo Bakelite, Tokyo, Japan). At 48 h after surgery, the exudative fluid was harvested from the closed continuous drainage system using a sterile technique. The fluid was centrifuged at 3,000 rpm (Fig. 1), and the supernatant was stored at −80° Celsius until analytical use. Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of TGF-beta1 and EMMPRIN. In addition, the concentrations of MMP-1, 2, 9, and TIMP-1 were quantified with ELISA in order to obtain fundamental data on the collagen metabolism. HA was also measured to clarify the effect of EMMPRIN.

The same postoperative rehabilitation management was applied to each patient. One sufficiently trained orthopedic surgeon (J. O.) examined each patient twice, before and 4 weeks after surgery, independent of the operating surgeon (K. Y.). The flexion angle was measured on a lateral radiogram of the knee, which was taken in the spine position under a manually maximal flexion force applied by the examiner. A relatively early time point of 4 weeks was chosen as follows: First, later time points were less desirable, because later ROM is strongly affected by various clinical and social factors in each patient's life and medical environment. Secondly, it is the ROM as observed at the earlier 4 weeks time point that contributes, together with the above factors, to the late stage ROM.

Statistically, a correlation between the preoperative personal factors and postoperative flexion angle was calculated, and a correlation between the postoperative knee flexion angle and the concentration of TGF-beta1 or

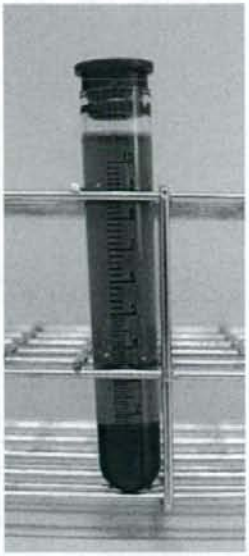

Fig. 1 At 48 h after surgery, the exudative fluid was harvested from the closed drainage system, and centrifuged at 3,000 rpm

EMMPRIN in the exudative fluid harvested at 48 h after surgery was then analyzed using the Pearson's product moment correlation coefficient. The significance level was set at $p = 0.05$. In addition, the power analysis [26] was made. Additionally, a correlation between the above-described EMMPRIN level and the MMP-1, 2, 9, TIMP-1 and HA levels in the same postoperative exudative fluid was analyzed in the same manner.

Patient demographics

The twenty patients included 3 men and 17 women with osteoarthritic knees of Grade IV in the Kellgren and Lawrence Scale. At the time of surgery, the age of patients averaged 72.2 years (range 57–84 years; SD 8.5), the mean weight averaged 59.0 kg (range 48.2–77.5 kg; SD 10.2), and the mean height averaged 1.46 m (range 1.41–1.70 m; SD 8.87). The preoperative knee flexion angle averaged 126.3° (range 90.0–145.0°; SD 13.6), while the femoro-tibial angle (FTA) measured on an antero-posterior radiogram taken during standing on one leg averaged 184.5° (range 172.0–196.0°; SD 6.8).

Surgical procedure

The surgery was performed by a senior surgeon (K. Y.) in the Hokkaido University Hospital according to the standard procedure [27, 44, 45]. Briefly, the distal femur and the proximal tibia were independently cut using the medial sub-vastus approach. The deep layer of medial collateral ligament was released from the tibia. The posterior part of the joint capsule was carefully detached from the tibia, while the PCL was preserved. In cases with marked varus deformity or flexion contracture of the knee, the tibial attachment of the semimembranous tendon was completely released from the tibia. In all the patients, ligament balancing was successfully achieved so that almost full ROM was obtained during surgery. PMMA cement was used in all the knees for fixation of the tibial, femoral, and patellar components. Lateral retinacular release was not commonly performed to establish appropriate tracking of the patella. After closing the skin incision, the knee instability and the intraoperative ROM was manually tested, and bulky dressing was applied.

Postoperative management

The knee was not immobilized postoperatively. Active dorsal and plantal flexion exercise of the ankle joint was encouraged immediately after surgery in order to avoid venous thrombosis. Straight leg raising exercise and isometric quadriceps muscle exercise started on the second day after surgery. Passive knee motion exercise was performed with a continuous-passive-motion machine. Active knee motion exercise was encouraged in sitting position. Walking exercise with crutches started several days after surgery, depending on the individual motion pain. Common physical therapy was applied thereafter. Manipulation of the knee was not performed in each patient.

ELISA for EMMPRIN, MMPs, TIMP-1, HA, and TGF-beta1

The EMMPRIN concentration was measured in exudative fluid using an ELISA that we developed previously [3]. Duplicate samples were assayed and mean values were analyzed. The results for all samples fell on a standard curve when the samples were five times diluted.

The assays for human MMP-1, MMP-2, MMP-9 and TIMP-1 were performed by ELISA kit (Daiichi Fine Chemical Co. Ltd., Toyama, Japan) by following manufacture's protocol. The sensitivity of each assay was 0.3 ng/ml for MMP-1, 0.24 ng/ml for MMP-2, 0.24 ng/ml for MMP-9, and 0.15 ng/ml for TIMP-1, respectively.

A modified N-acetylamino sugar estimation method was used for HA with Streptococcus purified hyaluronidase [13, 35] and assay was outsoused to Seikagaku Kogyo (Tokyo, Japan).

TGF-beta1 was quantified using Quantikine® TGF-beta1 ELISA kit (R&D Systems Inc. Minneapolis, MN, USA) according to the manufacturer's instructions. Concentrations

were calculated from the constructed linear standard curve. The sensitivity of the kit was less than 7 pg/ml TGF-beta1.

Western blot analysis for EMMPRIN

The samples were resolved by electrophoresis in a 15% SDS-polyacrylamide gel under reducing conditions as described [31]. The transferred membranes were incubated overnight at 4°C with goat anti-human EMMPRIN antibody (AF972, R&D Systems, Minneapolis, MN) diluted 1:500 followed by horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (Dako Japan, Kyoto, Japan) diluted 1:20,000. Additional details of the procedure were as described [31].

Results

The postoperative knee flexion angle ranged between 100° and 130° (the mean 116.8°; SD 10.1) at 4 weeks. The Pearson's correlation analysis revealed that the postoperative knee flexion angle was not significantly correlated with the age at the time of surgery ($r = -0.167$, $p = 0.486$), the body weight ($r = -0.095$, $p = 0.693$), the height ($r = -0.004$, $p = 0.988$), the preoperative knee flexion angle ($r = 0.325$, $p = 0.164$), or the preoperative FTA ($r = 0.042$, $p = 0.866$) (Table 1).

There was a significant positive correlation between the concentration of EMMPRIN and the knee flexion angle measured at 4 weeks ($r = 0.557$, $p = 0.0148$, $Z_{1-\beta}$ (power) = 0.74) (Fig. 2). The Western blot analysis showed a prominent band at 27 kDa corresponding to EMMPRIN in the exudate harvested after TKA (Fig. 3). The concentration of EMMPRIN was not significantly correlated with the concentration of MMP-1, 2, 9, TIMP-1, or HA (Table 2).

On the other hand, there was no correlation between the concentration of TGF-beta1 and the knee flexion angle measured at 4 weeks (Fig. 4).

Table 1 Correlation between the postoperative knee flexion angle and the other personal factors

Variable	r	p
Age	−0.167	0.486
Body weight (kg)	−0.095	0.693
Height (m)	−0.004	0.988
Preoperative knee flexion angle (degree)	0.325	0.164
Preoperative FTA (degree)	0.042	0.866

r = Correlation coefficient; p = p value

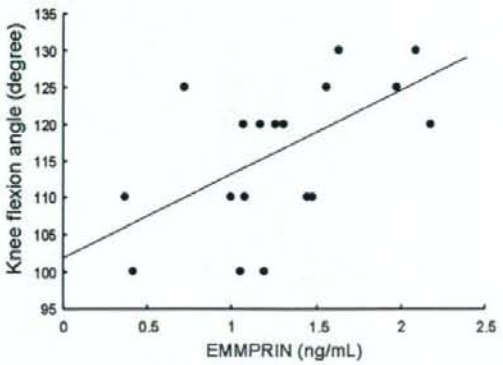

Fig. 2 There was a significant positive correlation ($r = 0.557$, $p = 0.0148$) between the concentration of EMMPRIN and the knee flexion angle measured at 4 weeks

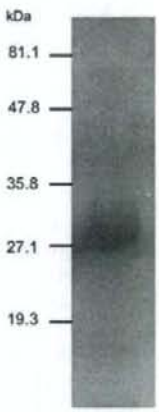

Fig. 3 The Western blot analysis showed a prominent band at 27 kDa for the EMMPRIN contained in the exudate harvested after TKA. *Left lane* molecular size marker

Discussion

The present study demonstrated that there was a significant positive correlation between the concentration of EMMPRIN in the exudate harvested from the joint at 48 h after surgery and the ROM measured at 4 weeks after TKA. Namely, there was a significant tendency that the ROM of patients having higher concentration value of EMMPRIN in the exudate was greater than that of patients having lower concentration value. In the present study, the ROM measured at 4 weeks was not significantly correlated with the other personal factors. Therefore, there is a high possibility that the concentration of EMMPRIN in the exudate is an independent variable affecting the ROM in the early phase after TKA. However, we should keep it in our mind that it is not clarified in this study whether EMMPRIN is a directly preventive factor against joint contracture in the present study.

Table 2 Correlation between the concentration of EMMPRIN and the concentration of MMP-1, 2, 9, TIMP-1, or HA

Concentration (ng/mL)*		r	p
MMP-1	199.10 ± 129.05 (range 22.2–507)	−0.305	0.1938
MMP-2	1,143.20 ± 311.07 (range 695–1,920)	−0.024	0.9227
MMP-9	1,729.65 ± 1,280.47 (range 625–6,400)	0.206	0.3888
TIMP-1	655.05 ± 204.15 (range 386–1210)	−0.291	0.2164
HA (mg/mL)	0.31 ± 0.51 (range 0.08–1.10)	0.349	0.2072

r = Correlation coefficient; p = p value

* Mean ± standard deviation

It has been reported that EMMPRIN promotes the differentiation of fibroblasts into myofibroblasts via induction of α-smooth muscle actin, independently of MMP induction during wound healing [18]. Myofibroblasts play an important role in the wound healing process [8, 16]. Therefore, we speculate that the early induction of EMMPRIN in surgically injured capsular and synovial tissues of the knee joint after TKA might accelerate the wound healing of the tissues, resulting in an increase of the postoperative ROM through the following possible mechanisms: (1) early wound healing might relieve the pain during knee motion exercise, enhancing the effectiveness of the postoperative rehabilitation [22]; (2) early completion of the healing process in these tissues might maintain an excellent ROM restored by postoperative knee motion exercise, because it might inhibit recurrence of contracture in the tissues.

EMMPRIN is also a potent inducer of MMP-1, -2, -3, -9, -14, and -15 by fibroblasts [19, 37, 41], and numerous studies have demonstrated a crucial role for EMMPRIN in regulating expression of MMPs [43]. The major function of MMPs is the degradation of extracellular matrices including collagens and proteoglycans. Therefore, we hypothesized that the concentration of EMMPRIN was correlated with MMP-1, 2, 9, or TIMP-1 in the exudate. In the present study, however, we could not find any significant correlation among them. It might not be surprising because MMPs are not regulated only by EMMPRIN but also by other factors. For example, several studies have recently reported that MMPs are rather overexpressed in the joint capsule with contracture [5, 14]. Thus, functions of MMPs in knee joint appear to be paradoxical. On the other hand, EMMPRIN induces production of HA in certain tumor cell lines via induction of hyaluronan synthases (HAS)-1, -2, and -3 [25]. Also, it has been reported that intraarticular administration of HA prevents joint contracture in experimental animal models [1]. Therefore, we assessed the concentration of HA in the knee exudate. In the present study, however, the concentration of HA was not correlated with the EMMPRIN concentration. Taken together, the role of EMMPRIN in the increase of ROM is complex, and further study is needed to elucidate the precise mechanism.

The concentration of soluble EMMPRIN in the exudate after TKA predominantly reflects EMMPRIN released from capsular and synovial tissues or infiltrated inflammatory cells within the knee joint as a result of either proteolytic MMP-dependent cleavage or shedding of membrane vesicles [38, 41]. However, the regulatory mechanism of EMMPRIN upregulation in those cells remain unknown. TGF-beta and epidermal growth factor are known to upregulate EMMPRIN expression in corneal epithelial cells [10] and in human breast epithelial cells in vitro [29]. Considering that TGF-beta is also associated with the regulation of extracellular matrix protein production [28], this factor might affect EMMPRIN expression in the knee joint after TKA. Because we have recently reported that EMMPRIN was upregulated by a component of basement membrane, laminin-111, there is a possibility that some particular extracellular matrix is an inducer of EMMPRIN [17]. In the future study, we should test these hypotheses. In addition, we should clarify whether there are other factors influencing the EMMPRIN expression.

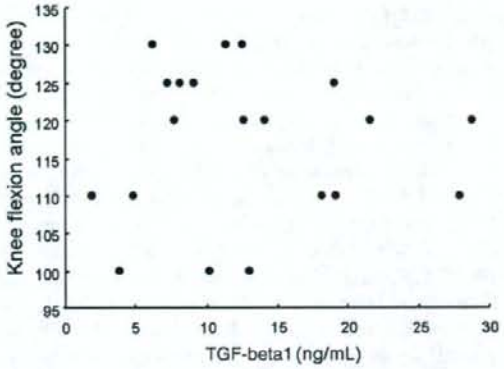

Fig. 4 There was no correlation ($r = 0.021$, $p = 0.930$) between the concentration of TGF-beta1 and the angle

In contrast to EMMPRIN, a significant correlation was not detected between the knee flexion angle measured at 4 weeks and the concentration of TGF-beta1 measured at 48 hours after TKA. However, this result does not necessarily exclude the role of TGF-beta1 expressed in the tissue surrounding the knee joint in the post-operative ROM, because we analyzed the relationship between the concentration of TGF-beta1 and only the ROM at 4 weeks. We should note that TGF-beta1 expressed in the joint capsule induces contracture of the joint [15, 32, 36]. Therefore,

there is a possibility that the concentration of TGF-beta1 measured at 48 h after TKA may influence the knee flexion angle measured at the time of 8 weeks or more after TKA.

There are some limitations in this study. First, the number of patients was only 20, because this was the first clinically oriented molecular-biological study to detect possible biological factors affecting the ROM after TKA. The power of the analysis on correlation between the concentration of EMMPRIN and the ROM in the present study was 0.74, because the correlation coefficient in the 20 samples was relatively high ($r = 0.557$). However, when we assume that a correlation coefficient of the population is 0.4, we need a total of 50 patients to have a power value of 0.8. Secondly, this study focused on the ROM only at 4 weeks after TKA because of the above-described reasons. Thirdly, we did not clarify the mechanism why the high EMMPRIN expression affected on the postoperative ROM. The fourth limitation is that we did not measure other possibly causative biological factors except for EMMPRIN and TGF-beta1 in the present study. The fifth limitation is the absence of EMMPRIN and TGF values in joint exudate before TKA and the absence of a matched control group. Beyond these limitations, however, the above-described results in the present study suggest that EMMPRIN expressed in the synovial and capsular tissues affects the postoperative ROM after TKA, as one of the biological factors in TKA.

As to clinical relevance, EMMPRIN in the exudate after TKA is a promising biological indicator to predict difficulty in restoring postoperative ROM. If such a predictable indicator in the exudate will be established, it will be useful to avoid serious loss of knee motion by applying a specific rehabilitation protocol to a patient who is predicted to suffer from joint contracture after TKA. For example, when we will find a patient having very low level expression of EMMPRIN in the exudate harvested immediately after TKA, we can strongly encourage the patient to perform knee motion exercise under analgesic treatment to a sufficient degree, or we can add joint manipulation under anesthesia to the above-described exercise protocol. However, further studies are needed to determine whether EMMPRIN is really a clinically useful indicator. A clinically oriented molecular-biological study using a large number of patients should be conducted in the near future to clarify the relationship between various biological factors and the postoperative ROM after TKA. Also experimental studies with animals should be conducted in the near future to clarify the effect of enhancing EMMPRIN expression on ROM after TKA. If EMMPRIN will be proven to be a directly preventive factor against joint contracture in the future, there is a possibility that we will be able to develop a new preventive measure to increase postoperative ROM by enhancing EMMPRIN expression in the joint.

In conclusion, there was a significant positive correlation between the concentration of EMMPRIN in the exudate harvested from the joint at 48 h after surgery and the ROM measured at 4 weeks after TKA. This study suggested that the postoperative ROM after TKA is affected not only by the mechanical and therapeutic factors but also by biological factors affecting collagen metabolism in the tissues surrounding the knee joint.

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Conflict of interest statement None.

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THORAX

Relationship between improved airflow limitation and changes in airway caliber induced by inhaled anticholinergics in chronic obstructive pulmonary disease

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Abstract

Rationale: Although airflow limitation improved by inhaled anticholinergic drugs varies among individuals with chronic obstructive pulmonary disease (COPD), the relationship between actual bronchodilation and improved pulmonary function and where in the lung such bronchodilation occurs remains unknown.

Objectives: To determine the relationship between improved pulmonary function and changes in airway caliber at various sites among airways in response to inhaled anticholinergics in patients with COPD, using 3-dimensional computed tomography (CT).

Methods: We performed CT at deep inspiration and detailed pulmonary function tests before and 1 week after daily inhalations of tiotropium bromide in 15 patients with clinically stable COPD. We analyzed the airway luminal area at the 3rd (segmental) to the 6th generations of 8 bronchi in the right lung.

Measurements and main results: Bronchodilation was demonstrated as an overall average of a 39% increase in the inner luminal area, and the mean forced expiratory volume in 1 sec (FEV_1) increased from 1.23 ± 0.11 to 1.47 ± 0.13 (SE). The magnitude of bronchodilation closely correlated with improved pulmonary function, particularly with that of FEV_1 ($r = 0.843$, $p < 0.001$). Such correlations were significant at the 4th to the 6th, but not at the 3rd generation of bronchi, and the slope of regression lines became steeper from the 3rd to the 6th generation.

Conclusions: Inhaled anticholinergics induce overall bronchodilation in proportion to improvements in FEV_1 in patients with COPD, and bronchodilation at distal, rather than proximal airways is the determinant of functional improvement.

Word Count: 244 words

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation that is not fully reversible. It is caused by a mixture of abnormal inflammatory responses in small airways and parenchymal destruction of the lungs, the relative contributions of which vary among individuals.[1]

Bronchodilator medications are central to the symptomatic management of COPD, although the magnitude of bronchodilation varies widely.[1-3] Factors that determine inter-subject variation in the bronchodilator response include pre-bronchodilator values of forced expiratory volume in 1 sec (FEV_1),[2-4] smoking status,[2, 5, 6] and β_2 adrenergic receptor gene polymorphism.[6] However, they account for only a small portion of the response. All classes of bronchodilators are thought to function by altering airway smooth muscle tone, and improvements in expiratory flow should reflect widening of the airways rather than changes in lung elastic recoil.[1, 7] However, the relationship between actual bronchodilation and improved pulmonary function and also where in the lung such bronchodilation occurs in patients with COPD have never been visually demonstrated. Moreover, bronchodilatory heterogeneity in the lungs has not been proven by any modality.

We designed a program to measure airway dimensions on computed tomography (CT) using the algorithm of curved multiplanar reconstruction. This allows visualization and accurate analysis of longitudinal bronchi and short axes of airways perpendicular to the long axis located anywhere in the lung.[8] We used our software to analyze airways with dimensions up to the 6^{th} generation of bronchi with an average inner diameter of 2.3 mm, and demonstrated that airflow limitation in patients with COPD is more closely linked to the dimensions of distal than proximal airways.[8]

Here, we attempted to measure the magnitude of bronchodilation (airway widening) at various sites throughout the lungs of 15 patients with moderate to severe COPD in response to daily inhalations of tiotropium bromide for 1 week. This compound is an effective bronchodilator with action that persists for over 24 h.[9, 10] We initially investigated the relationship between the overall magnitude of bronchodilation evaluated by 3-dimensional CT and improved pulmonary function parameters among the patients, and then explored the relationship at each generation of bronchi and/or at each lobe. Finally, we classified the subjects as good or poor responders to inhaled anticholinergics, and compared bronchodilatory behavior between the 2 groups.

METHODS

We recruited patients with clinically stable COPD (M/F, 13/2; age, 53 - 83 y; mean, 69.4 y) at Hokkaido University Hospital between September 2006 and March 2008. The diagnosis of COPD was confirmed based on the spirometric criteria of the GOLD guidelines, namely the post-bronchodilator ratio of FEV_1: forced vital capacity (FVC) of < 0.7.[1] All patients were either current or former smokers with an average smoking history of 61.8 ± 31.8 pack-years. The exclusion criteria for selecting participants in this study are described in the online supplement. Bronchial asthma was excluded based on clinical history and laboratory findings, including levels of IgE and/or eosinophils in the blood and/or sputum. All of the patients provided written informed consent to participate and the Ethics Committee for Human Research at Hokkaido University School of Medicine approved the study.

At the first visit, the patients were instructed to refrain from using any respiratory medication including tiotropium bromide for the next week. At the second visit for baseline measurement, we confirmed that the patients were stable and free of medication that might influence the spirometric data. We then conducted lung CT followed by pulmonary function tests. We acquired CT images while the patient held their breath at deep inspiration. Spirometric measurements were obtained using a rolling seal spirometer, the diffusing capacity of carbon monoxide was measured using the single breath method, and lung volumes were measured using a method of closed-circuit helium dilution that met all of the Japanese Respiratory Society (JRS) guidelines.[11]

From the following day, all of the participants inhaled tiotropium bromide once every morning for 1 week, which is sufficient to induce an effect.[12] At the third visit, which was precisely 7 days after the second, we confirmed that the participants had last inhaled tiotropium about 2 h previously and we measured lung CT and pulmonary function as described above once again.

Computed tomography and airway analysis

We used 2 multidetector-row spiral CT scanners, one with 4 (SOMATOME plus Volume Zoom, Siemens, Berlin, German), and the other with 64 (Aquilion Multi, TSX-101A/HA; Toshiba Medical Systems, Tokyo, Japan) detector arrays, because we have several CT scanners at our hospital. The same CT model was used to obtain images from specific individuals to avoid bias due to different types of CT scanners. We performed a validation study using phantoms to confirm the acceptability of data using both types of CT scanners for this study (online supplement), as we had for a Siemens scanner in our previous study.[8] We measured only the inner luminal area and not the airway outer wall.

Data acquisition parameters for both CT scanners are shown in the online supplement. We selected 3 upper, 2 middle and 3 lower bronchi from the right lung and analyzed the inner luminal area at 4 sites in each bronchus from the 3rd to the 6th generation, for a total of 32 sites per patient. Airway measurements are detailed in our previous publication and also in the online supplement. We measured lung volumes using 3D-CT volumetric analysis to confirm that CT images were

acquired from each patient at similar lung volumes before and after tiotropium bromide inhalation. This is because we were afraid that the difference in the lung volume on 2 occasions might significantly bias airway caliber, thus being potentially a confounding factor in this study. Details of these measurements are shown in the online supplement.

Statistics

Data are shown as means ± standard error of the mean (SEM), including the airway inner luminal area at various sites, because the distribution of normality for most of the results was not rejected by the Shapiro-Wilk test. We used paired Student's t tests to analyze differences in mean values between baseline and post-bronchodilator values, and unpaired Student's t tests for values between 2 groups. Relationships between 2 quantitative variables were examined using the Spearman test. We applied the Jonckheere-Terpstra test to examine trends in the %increase in the inner luminal area of 3rd to 6th generation airways. In addition, the groups were compared using the Kruskal Wallis test for multiple comparisons followed by the Mann-Whitney test. All statistical tests were 2-sided and values of $p < 0.05$ were considered statistically significant. Data were analyzed using SPSS for Windows version 12.0 software (SPSS Japan, Tokyo, Japan).

RESULTS

Airway measurements in patients with COPD before and after inhaled tiotropium

Table 1 shows the results of pulmonary function tests on 2 occasions.

Table 1. Pulmonary function tests before and after tiotropium inhalation for 1 week.

Pulmonary function tests	Baseline		One week after tiotropium bromide inhalation		P-value†	
VC, l (%predicted) *	3.41 ± 0.16	(107.8 ± 3.5)	3.80± 0.15‡	(120.3 ± 2.9‡)	< 0.001	< 0.001
IC, l	2.17± 0.13		2.4 ± 0.14‡		0.015	
FVC, l (%predicted)	3.25 ± 0.17	(102.7 ± 3.7)	3.71 ± 0.16‡	(117.4 ± 3.4‡)	< 0.001	< 0.001
FEV_1, l (%predicted)	1.23±0.11	(53.2 ± 4.2)	1.47±0.13‡	(63.7 ± 5.4‡)	< 0.001	< 0.001
FEV_1/FVC, %	0.38 ± 0.03		0.40 ± 0.04		0.056	
MMF, l/sec	0.44 ± 0.06		0.57 ± 0.09‡		0.002	
DL_{CO}, ml/min/mmHg (%predicted)	12.5 ± 1.4	(75.9 ± 7.1)	13.0 ± 1.5	(79.6 ± 7.9)	0.199	0.139
DL_{CO}/VA, ml/min/mmHg/l (%predicted)	3.06± 0.38	(68.9 ± 8.1)	3.11± 0.39	(70.0 ± 8.2)	0.540	0.507
TLC, l (%predicted)	6.51± 0.31	(120.1 ± 3.7)	6.61± 0.30	(122.2 ± 3.5)	0.120	0.109
FRC, l (%predicted)	4.07± 0.26	(124.3 ± 6.2)	3.97± 0.26	(121.5 ± 6.4)	0.263	0.286
RV, l (%predicted)	3.12± 0.23	(153.9 ± 9.5)	2.82± 0.22‡	(139.3 ± 9.1‡)	0.004	0.003
RV/TLC, %	47.3± 2.1		41.9± 1.8‡		< 0.001	

VC, vital capacity; IC, inspiratory capacity; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 sec; MMF, maximum mid-expiratory flow rate; DL_{CO}, carbon monoxide diffusing capacity; VA, alveolar volume; TLC, total lung capacity; FRC, functional residual capacity; RV, residual volume.
Data are shown as means ± standard error of mean. * Values in parentheses represent %predicted;
† Before vs. after tiotropium inhalation. ‡ $p < 0.05$ after tiotropium inhalation versus baseline.

Tiotropium inhalation for 1 week induced statistically significant increases in vital capacity (VC), FVC, inspiratory capacity (IC) and FEV_1, as well as decreases in residual volume (RV) and RV/total lung capacity (TLC).

Figure 1 shows 2 curved multiplanar reconstructed images of the posterior basal bronchus of one individual before and after tiotropium inhalation. We obtained short axis images perpendicular to the long axis from the 3rd to the 6th generation at very similar sites on both occasions. The visual evidence shows that bronchodilation indeed occurred after bronchodilator inhalation in this particular airway. However, we could not obtain clear airway images of the middle lobe from 1 patient and of the lateral basal bronchus from 2 others, because 1 had middle lobe syndrome, and the others had significant amounts of sputum within that bronchus. Table 2 shows the absolute values of the inner luminal area at 32 measurement sites before and after bronchodilator inhalation, as well as the % increase in the inner luminal area with treatment, which was calculated separately for the lobe and for the airway generation on average. Assuming that the airway lumen is a true circle, then the inner diameters (Di) calculated as $Di = 2\sqrt{Ai/\pi}$ at baseline were 4.5 ± 0.2, 3.3 ± 0.1, 2.7 ± 0.1 and 2.1 ± 0.1 mm at generations 3, 4, 5 and 6, respectively.

Table 2. Measurements of inner luminal area (mm^2) at sites of 8 bronchi before and after inhalation of tiotropium bromide.

	Generation of bronchi								
	3rd		4th		5th		6th		Averaged % increa
	Baseline	After	Baseline	After	Baseline	After	Baseline	After	in Ai / lobe, %
Right upper lobe									
Apical (B1)	21.6 ± 3.0	26.7 ± 3.5‡	10.0 ± 1.1	12.2 ± 1.3‡	6.3 ± 0.7	8.0 ± 0.9†	3.7 ± 0.5	5.2 ± 0.4‡	
Posterior apical (B2)	17.0 ± 1.6	22.0 ± 1.7‡	8.7 ± 1.0	12.4 ± 1.0‡	5.4 ± 0.7	7.1 ±0.5	3.2 ± 0.3	4.7 ± 0.4‡	**41.8 ± 7.9**
Anterior apical (B3)	24.2 ± 3.1	29.5 ±2.9‡	11.8 ± 1.4	15.3 ±1.4‡	8.2 ± 1.2	9.3 ±1.0	4.6 ± 0.5	5.7 ±0.6	
Right middle lobe									
Medial (B4)	11.0 ± 1.0	14.3 ±1.1‡	5.8 ± 0.5	7.7 ±0.6‡	3.7 ± 0.3	5.3 ±0.4‡	2.8 ± 0.3	4.2 ± 0.4‡	**39.1 ± 11.6**
Lateral (B5)	20.5 ± 2.5	22.3 ±2.1	11.3 ± 1.3	12.4 ± 1.0	6.9 ± 0.9	8.3 ± 0.7	4.7 ± 0.5	5.6 ± 0.4	
Right lower lobe									
Anterior basal (B8)	12.9 ± 1.9	17.0 ± 1.7‡	7.2 ± 0.9	9.6 ± 1.3†	5.2 ± 0.6	5.8 ± 0.6	3.6 ± 0.4	4.3 ± 0.4†	
Lateral basal (B9)	13.0 ± 1.5	15.7 ± 1.4†	7.1 ± 1.1	8.3 ± 0.9	4.7 ± 0.7	5.6 ± 0.7	3.0 ± 0.3	4.0 ± 0.4†	**37.8 ± 9.4**
Posterior basal (B10)	15.4 ± 1.3	19.6 ± 1.9‡	10.7 ± 1.4	13.2 ± 1.6†	7.1 ± 0.9	8.2 ± 1.0	4.6 ± 0.6	5.5 ± 0.7	
Averaged % increase in Ai/generation, %	**32.5 ± 4.8**		**38.4 ± 6.4**		**38.0 ± 9.1**		**48.0 ± 11.2**		**39.3 ± 7.2 ***

Data are shown as means ± standard error of mean (SEM). †$p < 0.05$ after tiotropium inhalation versus baseline.
‡ $p < 0.01$ after tiotropium inhalation versus baseline. *Average of all measurements

The magnitude of bronchodilation varied considerably among individuals at any site as reflected by relatively large SEM values. However, bronchodilation was statistically significant at 20 of 32 sites of the bronchi when we averaged the measurements from 15 patients.(Table 2) Generally, as the generation number increases, that is, as the size of the airways decreases, the average amount of bronchodilation appears to increase. However, the trend did not reach statistical significance. The average bronchodilator response was very similar among the lobes.

We then averaged all 32 measurements for each individual and compared the %increase in bronchodilation and the %improvement in pulmonary function tests among the participants. (Table 3)

Table 3. Relationship between % increase in inner luminal area and % improvement in pulmonary function parameters.

Pulmonary function tests	r value	p-value
VC	0.675*	0.006
IC	0.575*	0.025
FVC	0.639*	0.010
FEV_1	0.843*	<0.001
MMF	0.111	0.694
DL_{CO} / V_A	-0.157	0.576
TLC	0.164	0.558
FRC	-0.157	0.576
RV	-0.561*	0.030
RV/TLC	-0.646*	0.009

VC, vital capacity; IC, inspiratory capacity; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 sec; MMF, maximum mid-expiratory flow rate; DL_{CO}, carbon monoxide diffusing capacity; VA, alveolar volume; TLC, total lung capacity; FRC, functional residual capacity; RV, residual volume.

Overall bronchodilation significantly correlated with improvements in pulmonary function parameters, including VC, FVC, IC, FEV_1, RV, and RV/ TLC. Figure 2 shows that the change in FEV_1 correlated most closely with overall bronchodilation with a correlation coefficient of r =