て行った。研究参加は参加を許諾した場合でも拒否した場合でも全く同質の治療が行われることを説明した上で、患者の任意によりインフォームドコンセントを得て行った。

C．研究結果

FS-PDWIでは軟骨の形態をとらえることに関しては良好なコントラストが得られたが、いずれの染色においても染色性によるgradeによって信号強度に有意差を認めなかった。

FS-T2WIではaggrecan免疫染色でのみ信号強度に有意差を認め、(±)群（172.5±102.2）、(−)群(223.1±91.0)と、(−)群で有意に高い信号強度を示した(p<0.05)。

T2 mappingでは、safranin0染色で(+)群（42.1±6.4）、(−)群(69.7±14.6)と、(−)群で有意に高い信号強度を示し(p<0.05)、typeX collgenの免疫染色で(+)群（51.7±11.7）、(−)群(42.5±8.4)と、(+)群で有意に高い信号強度を示した(p<0.05)、また、aggrecan免疫染色で(±)群(42.9±8.5)、(−)群(52.5±11.3)と、(−)群で有意に高い信号強度を示した(p<0.05)
その他の群間では統計学的有意差を認めなかった。

D．考察

軟骨変性の形態的評価にはFS-PDWIは有用であったが、定量的評価においてはFS-PDWI,よりもFS-T2WI, T2 mappingの方が有用であった。safranin0染色の染色性の低下や、typeX collagen免疫染色陽性、aggrecan免疫染色陰性となった変性軟骨の特徴を示す部位においてT2値は有意に延長していた。T2値の延長は軟骨変性の存在を示唆するものと考えられた。

3.0T-MRI によるT2 mappingは軟骨変性の定量的評価に適しており、早期診断や治療評価に有用である可能性が示唆された。

E．結論

FS-PDWIで関節軟骨と軟骨下骨、半月板の全体像を評価した後、T2 mappingで軟骨変性の定量的評価を行うことにより、3.0T-MRIが関節軟骨変性のの早期診断と治療評価に臨床応用可能であると考えられた。

G.研究発表

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日本関節鏡・膝・スポーツ整形外科学会雑誌　vol.35 No.1 2010

2.学会発表

1）久保晴司ほか　3T-MRIにおける関節軟骨変性の定量的評価－病理組織像との比較
JOSKAS 札幌　2009 6 26

H．知的財産権の出願・登録状況

1. 特許取得
記載事項なし
2. 実用新案登録
記載事項なし
3.その他
記載事項なし

厚生労働科学研究費補助金（長寿科学総合研究事業）
（総括・分担）研究報告書

MR像による関節不整度の実践と確立

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整形外科学　助教

研究要旨
MRIを用いた変形性膝関節症（膝OA）の客観的な重症度評価法を確立すること目的とした。膝OAでは疾患の進行に従い、MRI上、大腿骨顆部の輪郭の不整度が増大する。この不整の程度を測定するソフトウェアを独自に開発し、計測値を不整度指数と称した。これまでの研究において不整度指数と臨床症状が相関することを示してきた。今回は以下の二つの研究を施行した。研究１：ソフトウェアをバージョンアップし不整度計測をより精密に実施可能とした。また、不整度を表すことが可能である複数あるパラメータのうち、最も重症度を判定するのに適したパラメータが何であるかを検討し、輪郭の幅のばらつきが最もよいパラメータであることを明らかにした。
研究２：不整度が高いということは組織学的には軟骨下骨に形成される嚢包様変化の密度が高いことをこれまでに明らかにしてきたが、この嚢包性変化が膝の疼痛に関連していることを、免疫組織学的に調べた。その結果、嚢包内には疼痛関連物質の発現が高くなっていることがわかり、症状発現に関連していることが示唆された。

A．研究目的

変形性膝関節症（膝OA）の客観的な重症度評価法を確立することが膝OAの治療体系の作成に必須であり、治療効果の判定のためにも必要とされている。本研究はMRIを用いた評価方法を確立することである。

研究１：MRI上みられる大腿骨顆部輪郭の不整度が膝OAの重症度を示す指標となり得ることをこれまでに示してきたが、そのためのソフトウェアを改良し、不整を表現すると考えられる複数のパラメータのうち至適なパラメータを決定すること。

研究２：不整度が高いということは組織学的には軟骨下骨に形成される嚢包様変化の密度が高いことをこれまでに明らかにしてきたが、この嚢包性変化が膝の疼痛に関連していることを、免疫組織学的に調べること。

B．研究方法

１．　内側型変形性膝関節症である55膝を対象とした。レントゲン分類としてKellgren&Lawrence分類を用い、膝の臨床症状をJKOMにて評価した。また、独自に開発した不整度を計測するソフトウェアにより、不整度を表現する４つのパラメータを計測した。レントゲン評価とJKOMの関係、４つのパラメータとJKOMの関係を統計学的に検討した。

2.内側型変形性膝関節に対してTKAが施行された15膝を対象とした。MRI・レントゲン検査により外側には疾患が及んでいない症例を選択した。
TKA施行時に得られた大腿骨顆部の組織標本を作製し、疼痛関連物質であるSubstance-P, TNF-alpha, Cox-2などに対する免疫染色を施行した。

C．研究結果

1．不整度計測はレントゲン分類に比し、膝の臨床症状を反映した。不整度をあらわすパラメータの中では輪郭の幅のばらつきが最もよく臨床症状を反映していた。

2．大腿骨内側顆では形成された嚢包内にSubstance-P, TNF-alpha, Cox-2, TUJ1の発現が確認されたが、外側顆では発現を認めなかった。Cox-1は内側外側ともに同程度発現を認めた。これらよりOA膝では軟骨下骨も疼痛を発生する場と考えられた。

D．考察

膝OAの重症度を客観的に示すには当該関節の画像検査が現時点では最も優れていると考えれらる。血中マーカーや尿検査では体内に多数存在する関節のどの変化を反映するものであるかを判定できないからである。現時点ではMRIが最も目的にかなった検査として期待されている。我々が提唱した大腿骨顆部の輪郭が不整度を数値化する方法もそのひとつであり、今回は輪郭幅のばらつきにより不整度を計測する方法が最も臨床症状を反映することがわかった。輪郭の不整をは軟骨下に生ずる病的変化を捉えているものと考えられる。今回の研究では、内側型変形性膝関節症では大腿骨内側顆の軟骨下骨に形成される嚢包性変化の中に疼痛関連物質の発現が強くなっていることがわかり、大腿骨外側顆では発現がみられないことがわかった。これは不整度測定の意義が免疫組織学的にも支持されたものと考えられる。高齢社会を迎え、高齢者のQOLに大きく影響する膝OAであるが、どの程度の膝にどういった治療法が適切であるかという目安はほとんどない。人工膝関節置換術（TKA）は年々増加し、2009年度には60,000件を超える手術が施行された。不整度はこの手術を施行する目安を数値で示すことなど、治療法の選択の指標となりえると期待できる。

E．結論

MRIを用いOA膝の客観的な重症度評価を可能とする方法を示した。本法で計測している指数は大腿骨顆部軟骨下骨の疼痛関連物質の発たを反映している。また、本法は治療法の選択の目安となりうる。

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H. 知的財産権の出願・登録状況
1. 特許取得
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3.その他
記載事項なし

Ⅲ. 研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ochiai N, Sasho T, Tahara M, et al.	Objective assessments of medial osteoarthritic knee severity by MRI: new computer software to evaluate femoral condyle contours	Int Orthop	Sep 9	Epub ahead of print	2009
久保晴司、黒田良祐、岩間祐基、松下雄彦、松本知之、藤井雅彦、杉村和朗、黒坂昌弘	3T-MRIにおける関節軟骨変性の定性的、定量的評価－病理組織像との比較	日本関節鏡・膝・スポーツ整形外科学会雑誌(JOSKAS)	vol.35	4-5	2010
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Ⅳ. 研究成果の刊行物・別冊

International Orthopaedics (SICOT)
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ORIGINAL PAPER

Objective assessments of medial osteoarthritic knee severity by MRI: new computer software to evaluate femoral condyle contours

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Abstract An irregular contour of the medial femoral condyle (MFC) on magnetic resonance imaging (MRI) appears to indicate the severity of medial-type knee osteoarthritis (OA). The purpose of this study was to establish a system to enable objective assessments of OA knee severity using newly developed software that semi-automatically measures irregularity of the MFC. (1) We evaluated 48 patients aged 50–83 years with 55 knees of medial-type OA. The following scores were recorded: Lysholm score, visual analogue scale (VAS) and the Japanese Knee Osteoarthritis Measure (JKOM). MFC irregularity was automatically calculated by newly programmed computer software. Four parameters for condyle irregularity were calculated: (a) the average thickness of the contour (ATC), (b) the ratio of the upper surface length to the lower surface length of the contour (RUL), (c) average squared thickness of the contour (ASTC) and (d) standard deviation of the contour thickness (SDC). (2) Nine knees that underwent total knee arthroplasty were further analysed histopathologically and compared with irregularity score. Statistically, the RUL and SDC were significantly correlated with the Lysholm score, VAS and JKOM, with good reliability. Histological examinations showed that an irregular contour reflected the density of cystic lesions formed in subchondral bone. An irregularity of MFC on MRI is correlated with OA disease severity clinically and histopathologically. The new computer software is useful to objectively assess OA disease severity.

Introduction

Osteoarthritis (OA) of the knee joint is a common joint pathology. X-ray examination is generally performed to assess the status of the OA knee joint, as it identifies the characteristic features of osteophytes, thickening of subchondral bone, cyst formation, malalignment and reduction of joint space due to cartilage loss. Among the indices to assess severity of OA using X-ray, the Kellgren-Lawrence (K/L) scale [1, 2] is frequently used and believed to be mostly reliable. However, its actual relationship with clinical severity of OA is controversial.

Magnetic resonance imaging (MRI) has also been used to evaluate OA knee joints due to its ability to detect cartilage abnormalities, osteophytes, subarticular cysts, bone attrition, synovitis, meniscal degeneration, bone marrow oedema, joint effusion, intra-articular loose body and periarticular cysts [3]. However, the clinical meanings of these abnormalities are not fully defined. One reason for this might be that the chief complaint of most patients is not

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deformity but knee pain, and the pain mechanisms of OA are not fully understood.

Recent evidence suggests that the subchondral bone is important for both pain origination and OA progression [4–6]. On MRI, contours of the femoral condyle or the tibial plateau are often depicted with irregularities, which could correspond to thickening of subchondral bone seen on X-ray [7]. In a pilot study, we reported that irregularities of the femoral condyle on MRI and knee functional scores were inter-related. In that study, calculation of irregularity was done with software that was developed for another purpose [8]. This result prompted us to develop new computer software that exclusively measures the irregularity of the femoral condyle.

Our hypothesis is that an irregular contour of the femoral condyle on MRI could be an indicator of OA disease severity and that measuring the irregularity of the contour should enable us to objectively assess OA severity. The purpose of this study was to develop new computer software that could exclusively measure the irregular contour of the femoral condyle and establish a reliable index that reflected OA severity. The relationship between OA clinical severity and femoral condyle irregularity was examined. Histological examination was performed as well.

Materials and methods

Patients and clinical assessments

Forty-eight consecutive patients with a total of 55 knee joints were recruited for this study. Patients with a history of trauma, previous surgery or inflammatory arthritis were excluded. Patients' mean age was 72.8 (range 50–83) years. None had prior surgical treatment. Informed consent was obtained from all patients after the nature of the examinations had been fully explained. All examinations were performed in accordance with the rules and regulations of the local human research committee. All patients were clinically examined at their first visit. They were scored using the Lysholm score [9], visual analogue scale [(VAS) on a 100-cm scale: 0 = no pain; 100 = most severe pain] and the Japanese Knee Osteoarthritis Measure (JKOM) [10]. Lysholm scores without items for instability were used. The JKOM is a counterpart of the Western Ontario and McMaster Osteoarthritis Index (WOMAC) that was developed for assessing Japanese OA patients by taking the Japanese lifestyle into account. It has proved to be as good as or better than the WOMAC or the Medical Outcomes Study 36-Item Short-Form Health Survey (SF-36) in terms of its reliability and validity [10].

Imaging

All patients underwent MRI of the affected knees within two weeks of their first visit. MRI was performed with a 1.5-T scanner (Signa, GE Medical Systems) equipped with a knee surface coil. The sequence used in this study was sagittal fast spin echo (FSE) proton density (TR 2,000 ms, TE 16 ms, field of view 14–16 cm, matrix 512 × 512, number of excitations = 2, and slice thickness = 4 mm without an interslice gap). Three sagittal slices that represented the centre of the medial femoral condyle (MFC) were selected for analysis by two orthopaedic surgeons who reached a consensus without knowledge of patient age or sex. The lateral femoral condyle (LFC) was assessed as well. X-ray grading employing the K/L scale [1] on weight-bearing radiographs was also recorded.

Software interface

Irregularity of the contour of the femoral condyle was calculated by computer software that was programmed in MATLAB6.5 (Cyber Net Systems, Tokyo). Digital Imaging and Communication in Medicine (DICOM) data can be incorporated directly with this software. The procedures used to measure the irregularity of the contour of the MFC were as follows: (1) Images to be assessed were selected. Images that represented the centre of the medial compartment were selected. Usually, six or seven images were taken for a medial compartment and three slices that represented the centre of the compartment were selected. (2) The image was converted to black and white (Fig. 1b). The threshold between black and white was determined by the histogram of the dots comprising images. With the vertical axis defined as the number of dots and the horizontal axis as the luminescence of dots, the histograms of these images usually consisted of two or three peaks. The threshold was determined manually so as to leave only the first peak. This meant that only very low luminescence dots remained; i.e., only very black dots. (3) The contour of the MFC was extracted (Fig. 1c), after which the upper and the lower surfaces of the extracted contour were automatically traced (Fig. 1d). Using these two lines, four parameters reflecting irregularity were automatically calculated: (a) average thickness of the contour (ATC), (b) ratio of the length of the upper surface to the lower surface length of the contour (RUL), (c) average squared thickness of the contour (ASTC) and (d) standard deviation of contour thickness (SDC). Theoretically, the more obvious the irregularity or thickening becomes, the larger the ATC, RUL, ASTC and SDC values become.

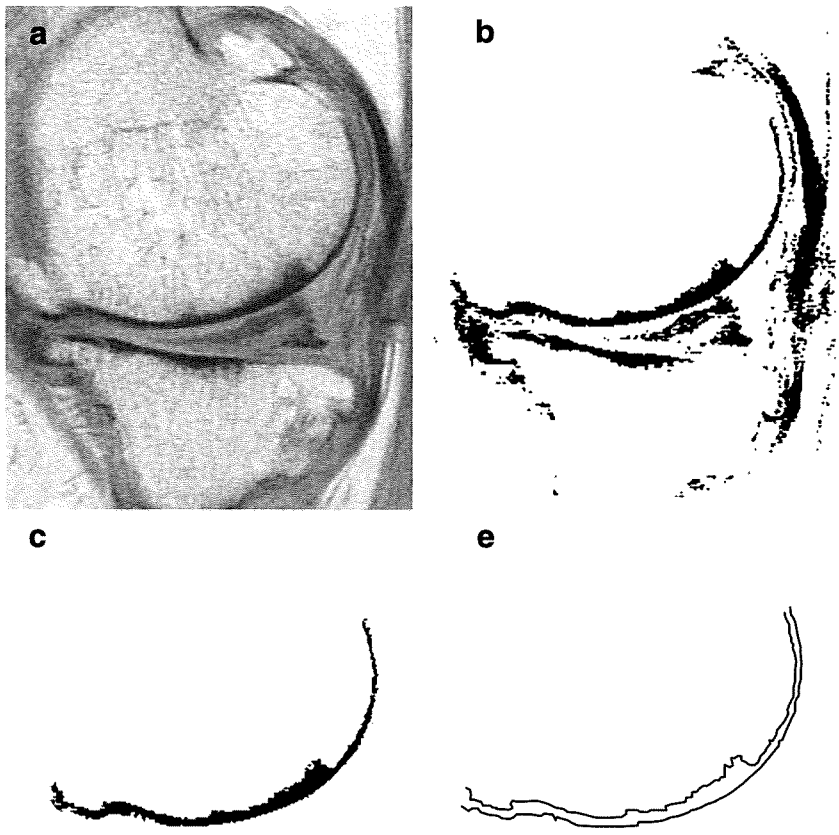

Fig. 1 Procedures to calculate irregularity of the condyle. **a** Image to be assessed is selected on the personal computer (PC). **b** The image is then converted to black and white. **c** Only the condyle contour is extracted. **d** The upper and lower surfaces of the extracted contour are traced, making two lines. Using these two lines, parameters reflecting irregularity are calculated

Interobserver and intraobserver reliabilities

Using ten randomly selected knees of the 55 knees evaluated, intra- and interrater reliabilities, intraclass correlation coefficient (ICC) (1, 1) and ICC (2, 1) were determined.

Specimens and histological evaluation

Nine knees that underwent total knee arthroplasty (TKA) following MRI assessments were used for this study. At the time of TKA, the weight-bearing areas of the MFC and the LFC were obtained. Specimens were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline for 24 h. The MFC and LFC were demineralised in 20% ethylenediaminetetraacetic acid (EDTA) at room temperature for six weeks. They were then embedded in paraffin. Sagittal sections (6 μm) were made and mounted on glass slides. Following staining with Mayer's haematoxylin solution and 1% eosin alcohol solution (H&E staining), the total number of cystic lesions that invaded the subchondral bone plate or calcified zone were counted from three slides that corresponded to weight-bearing areas. Density of cystic lesions was expressed as the number of cystic lesions per 10-mm-length of specimen [11]. Correlation of parameters related to irregularity of the contour on MRI and the density of cystic lesions were examined.

Correlation of contour irregularity and clinical assessments

Correlations between parameters related to irregularity and the clinical score or the numbers of cystic lesions were analysed using Pearson's correlation coefficient with Statview 4.1 (Abacus, Berkeley, CA, USA). The p value was obtained from an analysis of variance, and statistical significance was defined as $p<0.05$.

Contour irregularity and clinical assessments of each K/L group

Post hoc test (Bonferroni/Dunn) was used for comparing contour irregularity and clinical assessments among each K/L group (Statview 4.1) where $p<0.05$ or 0.01 was considered significant.

Results

Interrater and intraobserver reliabilities

Interrater reliabilities were ATC=0.463, RUL=0.811, ASTC=0.384, and SDC=0.891. Intrarater reliabilities were ATC=0.573, RUL=0.834, ASTC=0.342, and SDC=0.923. (Measurements are defined in Materials and Methods.)

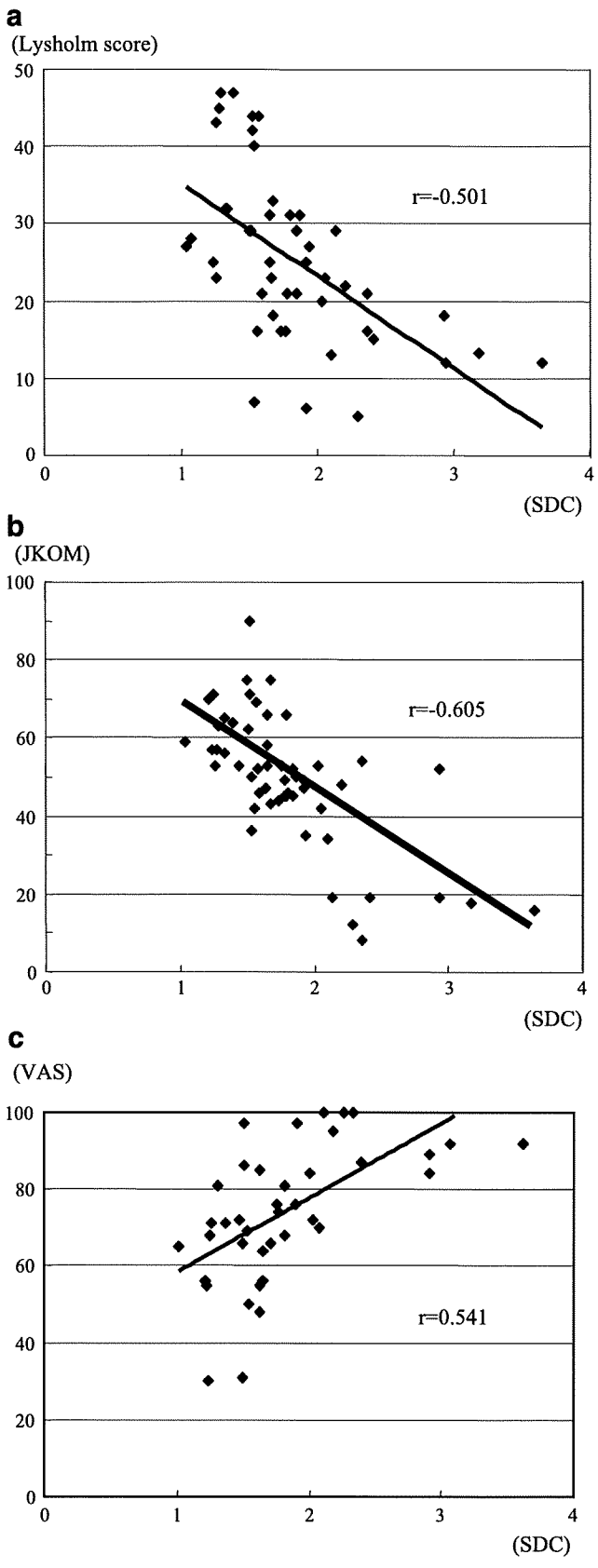

Fig. 2 Relationships between the standard deviation of the contour thickness (SDC) values and knee scores. The SDC scores are negatively correlated with **a** the Lysholm scores and **b** the Japanese Knee Osteoarthritis Measure (JKOM) scores, whereas **c** the SDC and visual analogue scale (VAS) are positively correlated

Therefore, the RUL and the SDC were selected for further analysis.

Clinical scores and X-ray grading

The average Lysholm score was 25.65±11.01 (range 5–47), the average VAS was 74.03±18.03 (range 30–100) and the average JKOM was 49.33±18.81 (range 8–90). Thirteen, seven, 23 and 12 knees were classified as K/L I, II, III and IV, respectively.

Correlations between clinical assessments and contour irregularity

1. RUL and knee Scores

The RUL values and Lysholm scores were negatively correlated (r=−0.448, p=0.0016), as were the RUL and the JKOM (r=−0.623, p<0.0001). A significant positive correlation was found between the RUL and the VAS (r= 0.472, p=0.0021).

2. SDC and knee scores

The SDC values and the Lysholm scores were negatively correlated (r=−0.501, p=0.0003), as were the SDC and the JKOM (r=−0.605, p<0.0001). A significant positive correlation was found between the SDC and the VAS (r= 0.541, p=0.0003; Fig. 2).

Contour irregularity and clinical assessments of each K/L group

The RUL tended to rise as the K/L grade increased, but no significant difference was observed between the K/L I and II and the K/L II and III. The SDC also tended to rise as the K/L grade increased, but no significant difference was observed between the K/L II and the K/L III. As for clinical assessments, the Lysholm score, VAS, and the JKOM tended to change according to the K/L grading, but only the K/L I and the K/L IV showed significant difference in all the three assessments (Table 1).

Correlation between MRI findings and histopathological examination

Derangements of the architecture of the trabeculae bones and cystic lesions were observed in the subchondral bone of the MFC in all nine cases used for histochemical

Table 1 Irregularities and clinical scores of each K/L grade

Average +/-standard deviation

	RUL	SDC	Lysholm score	VAS	JKOM
K/L I	1.08+/-0.05	1.16+/-0.33	35.9+/-10.2	66.3+/-18.4	60.1+/-7.1
K/L II	1.13+/-0.07	1.68+/-0.36	26.0+/-6.9	67.0+/-2.8	50.5+/-12.0
K/L III	1.16+/-0.07	1.73+/-0.48	22.8+/-10.7	71.6+/-19.1	50.9+/-21.5
K/L IV	1.31+/-0.13	2.52+/-0.78	20.2+/-6.1	86.8+/-10.9	36.6+/-16.2

(**: p<0.01, *: p<0.05, ns: not significant)

K/L Kellgren and Lawrence grade, *RUL* ratio of the upper surface to the lower surface of the contour, *SDC* standard deviation of contour thickness, *VAS* visual analogue scale, *JKOM* the Japanese Knee Osteoarthritis Measure

examinations, whereas less formation of cystic lesions was seen in the LFC. Average density of cystic lesions was 7.6± 4 .4/10 mm in the MFC and 1.6±1.6/10 mm in the LFC. Representative H&E staining of subchondral bone of the MFC is shown in Fig. 3a. Correlation coefficients for density of cystic lesions and irregularity were as follows: RUL: r=0.446, p<0.01; SDC: r=0.846, p<0.0001. Thus, irregular contours and the numbers of cystic lesions were positively correlated (Fig. 3-b).

Discussion

Obtaining a reliable index that objectively reflects knee OA severity should be helpful for determining treatment options and may possibly be useful to assess the efficacy of interventions. Evaluation based on imaging of affected joints is ideal, as it is not affected by the pathology of other joints that might obscure the results of an OA biomarker, such as in a urine or blood sample. Among several imaging technologies, radiographic examination is the most commonly used. However, the reliability for evaluating OA severity based upon radiographic findings is controversial.

Several studies showed that the radiographic features of knee OA were significantly associated with pain [12–14], whereas other studies have reported that OA severity based on radiographic findings was independent of pain [15, 16]. Many patients with relatively minimally damaged joints report knee pain [15]. In fact, we sometimes encounter a discrepancy between the characteristic findings of OA on X-ray and the clinical severity of knee OA. Recently, MRI has increasingly been used to diagnose or evaluate OA.

The typical features that may have an association with joint pain are osteophytes [17], bone oedema [4] and synovitis [4, 18, 19]. However, the clinical importance of these findings has not as yet been confirmed [20–22]. Oedema in the subarticular bone marrow adjacent to the knee detected by T2-weighted MRI is associated with painful knee OA [4]. In contrast, a finding on MRI of subchondral bone oedema cannot satisfactorily explain the presence or absence of knee pain [20].

In this study, we focused on the MFC contour, which presumably corresponds to the subchondral bone of the MFC on MRI [7]. One of the radiographic features of OA is a sclerotic change of subchondral bone. Sclerotic lesions are a mixture of thin and thick subchondral bone as OA becomes more severe. In our previous study, the cystic lesions of sclerotic lesions were positively stained with antibodies against pain-related molecules, including cyclooxygenase-2 (COX-2) and tumour necrosis factor alpha (TNF-α) [23]. Thus, we speculated that the subchondral bone of the affected compartment could be a source of pain, and that the extent of corresponding pathological changes that occurred in subchondral bone could be assessed by newly developed software presented in this paper.

Our previous pilot study showed that irregularity of the MFC on MRI was correlated with knee scores [8]. In this

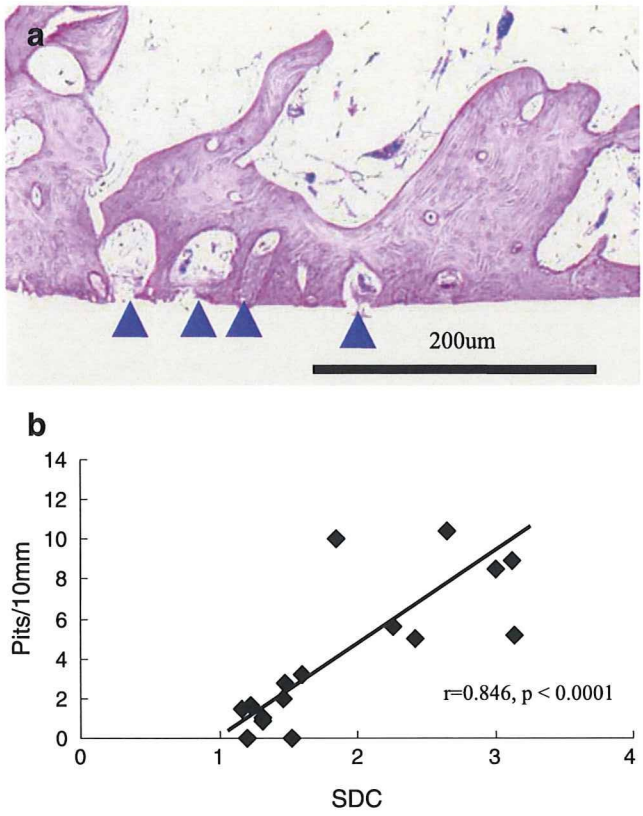

Fig. 3 Relationship between density of cystic lesions and irregularity. **a** Representative haematoxylin and eosin (H&E) staining of subchondral specimen retrieved at total knee arthroplasty (TKA) showed multiple formations of cystic lesions. **b** Density of cystic lesions and irregularity calculated according to preoperative magnetic resonance imaging (MRI) [standard deviation of the contour thickness (SDC)] are positively correlated

study, we present the possibility that RUL and SDC scores, which represent the irregularity of the MFC on MRI, could serve as indicators for disease severity in medial-type OA because the values of these two parameters increased as the Lysholm score and the JKOM decreased and as the VAS went up. Furthermore, these two parameters are positively correlated with the number of cystic lesions involved with the subchondral bone plate or the calcified zone. In this study, four parameters were initially employed, as the dependence upon only one or two values might miss detecting different types of irregular changes of the MFC. However, the ATC and the ASTC, which are related to changes in thickness, were far from being reliable. This was probably because a slight inconsistency in reproducing a black-and-white image during the process of conversion significantly affected these two parameters, especially the ASTC. We focused our attention on the femoral condyle and not on the tibial side in this study. There was a strong correlation between femoral and tibial cartilage volumes in the medial and lateral tibiofemoral joints both in subjects with normal knees and those with radiological OA [24]. Changes to the femur were sensitive enough, and for that reason, the femoral side was selected.

There are many methods for treating OA, from nonsurgical treatments such as nonsteroidal anti-inflammatory drugs, disease-modifying osteoarthritis drugs, rehabilitation and insoles, to surgical treatments such as arthroscopic debridement, osteotomy, arthrodesis, knee arthroplasty and others [25]. Choosing a treatment option is often difficult because there is no reliable objective index. Our next goal is to use this assessment system when selecting treatment options. For now, we can only say that when an irregularity of the MFC becomes large, TKA would be the only treatment, because a field of pain-generation area (i.e., COX-2 and TNF-α-positive subchondral bone area) will be removed at the time of TKA, so that treatments such as an arthroscopic technique or osteotomy to preserve these areas would fail. Further study will enable us to select suitable treatments based on the RUL and SDC values.

One of the limitations of this study relates to image accuracy. For high-quality image acquisition, three-dimensional techniques may be required, such as spoiled gradient-echo sequences [26] that would produce more reliable indices. Second, subjects in this study had apparent knee OA on X-ray. This technique is not useful for patients in the early stage of OA who only have cartilaginous damage and thus have no remarkable changes on X-ray. When evaluating these patients, other MRI protocols that can detect hyaline cartilage, such as T2 maps at 3 T [27] or contrast-material-enhanced techniques [26] have to be used. The problem with those methods is that they are too time consuming for daily clinical use where acquisition time is limited per patient. FSE proton density is the primary pulse sequence for MRI of the knee joint and is one of the suggested protocols in clinical work [28]. It only requires a short time for acquisition, so we routinely use those images in daily clinical work, and they were employed in this study.

Although MRI is a rather expensive investigation for assessment of OA severity, the assessment of contour irregularity could be a useful tool for patients when normal radiographs are not conclusive.

Conclusion

New computer software that assesses irregularity of the MFC contour is useful for evaluating medial-type OA knees. Objective values derived from this software are closely associated with knee scores and can be used as an indication for determining treatment options.

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Disclosures None.

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ORIGINAL ARTICLE

Detection of pain-related molecules in the subchondral bone of osteoarthritic knees

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Abstract Knee pain is predominant among osteoarthritis (OA) patients, but the mechanism is poorly understood. We investigated subchondral bone as a source of OA knee pain using immunohistochemistry. Fifteen medial-type OA knees with minimum involvement of the lateral compartment determined by X-ray as well as magnetic resonance imaging that received total knee arthroplasty (TKA) were involved. Each pair of the medial femoral condyle (MFC) and lateral femoral condyle (LFC) was compared obtained at the time of TKA. Osteocartilaginous MFC and LFC specimens were histologically examined and stained with antibodies against cyclooxygenase 1 (Cox-1), cyclooxygenase 2 (Cox-2), substance P, tumor necrosis factor-alpha (TNF-α), and neuron-specific class III beta-tubulin (TUJ1), a pan-neuronal marker. Formation of cystic lesions was more frequently seen in the MFC. The lesions were composed of vascular endothelial cells, osteoclasts, and mononuclear cells and were present in similar proportions between the MFC and the LFC. Four out of 15 MFC specimens were positive for Cox-1, 15 for Cox-2, and 13 for TNF-α. No LFC specimens were positive for any antibodies. Substance P-positive and TUJ1-positive fibers were found in the subchondral area of the MFC, but not in the LFC. Pathological changes in the subchondral bone can be a source of knee pain, which was detectable by the positive immunoreactivity of substance P, Cox-2, TNF-α, and TUJ1, in the subchondral bone of affected compartments. The relatively immediate reduction in pain obtained by TKA might account for the involvement of the subchondral bone in knee pain because most of the affected subchondral plate is excised in TKA (debridement effect of TKA).

Keywords Cox-2 · Knee osteoarthritis · Subchondral bone · Substance P · TNF-α · TUJ1

Introduction

The incidence of osteoarthritis (OA) of the knee has been increasing as society ages. OA of the knee is associated with substantial and persistent reduction of physical function in elderly people and can be disabling from the very onset [1, 2]. Therefore, establishment of an effective treatment for OA of the knee has become increasingly important in terms of social security as well as medical care. Among the many complaints that OA patients have, knee pain is the most common and predominant symptom. However, up until now, the mechanism of OA knee pain has been poorly understood.

It has been reported that OA knee pain originates from periarticular tissues and secondary synovitis [3, 4]. Some authors have reported that the joint pain mainly arises from free nerve endings that exist in the capsule or in the synovium [3, 5, 6]. Other joint components such as the periosteum, ligaments, menisci, muscle, and bone marrow have also been reported to be sources of OA knee pain [7–9]. A controversial area regarding the source of knee pain is

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when the cartilage and subchondral bone show drastic histological changes as OA progresses, since cartilage is aneural and subchondral bone is sparsely innervated [10, 11]. In 2007, Suri et al. reported on innervation of the osteochondral junction in human knee OA samples and indicated a possible contribution of the subchondral area to OA knee pain [12]. Our previous study showed that pathological changes in the subchondral plate were detectable by magnetic resonance imaging (MRI) as irregular contours of the femoral condyle [13]. We also showed that the irregularity became increasingly obvious as OA progressed and that an irregular change of the femoral condyle correlated with the knee score [14]. Taken together, these reports imply that the subchondral plate of the femoral condyle may be a potent source of pain in knee OA.

The purpose of the present study was to investigate the subchondral bone as a source of OA knee pain. We used immunohistochemical analysis to compare the medial femoral condyle (MFC) and the lateral femoral condyle (LFC) obtained from patients at the time of total knee arthroplasty (TKA). For this purpose, we selected patients with medial-type OA knees with minimal involvement of the lateral compartment.

Materials and methods

Patients

Included in this study were 15 medial-type OA knee patients who underwent TKA at our institution. At the time of operation, the patients' age and gender, as well as X-ray image grading of the medial and lateral tibiofemoral joints and MRI of the bone marrow edema (BME) of each compartment were recorded. For immunohistochemical analysis, patients with a lateral compartment less than grade II on the Kellgren and Lawrence (K/L) scale [15] and who displayed a lack of BME on MRI were selected. Patients with other arthritic diseases such as rheumatoid arthritis were excluded from the study. The study protocol was approved by the institutional ethics committee of Chiba University, and informed consent was obtained from all the patients.

X-ray and MR imaging

A standard anteroposterior X-ray was used to determine the K/L score for the medial and lateral compartments. Using MRI (Signa 1.5 T, GE Medical Systems), we assessed sagittal and coronal fat-suppressed T2-weighted images (TR 2,000 ms, TE 87 ms, field of view 13×13 cm, matrix 512×256, and 3 mm slice thickness with a 1-mm interslice gap) to detect BME in the affected knees

Specimens

At the time of TKA, the weight-bearing areas of the MFC and LFC were obtained (Fig. 1). Synovium was also obtained from the medial compartment. Specimens were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h. The femoral condyle was demineralized in 20% ethylenediaminetetraacetic acid at room temperature for 6 weeks and then embedded in paraffin. Sagittal sections (6 μm) were cut and mounted on glass slides.

Immunohistochemical staining

The sections were deparaffinized using 80% xylene and ethyl alcohol, rinsed with PBS (pH7.4), and stained with Mayer's hematoxylin solution and 1% eosin alcohol solution (H&E staining). For immunohistochemistry, sections were washed with PBS, soaked in 0.3% methyl alcohol to remove endogenous peroxidase activity (from blood cells), then incubated with the following antibodies: anticyclooxygenase 1 (Cox-1, 1:200, Catalog No. 160109, Cayman Chemical, Ann Arbor, MI, USA), anticyclooxygenase 2 (Cox-2, 1:200, Catalog No. sc-1745, Santa Cruz Biotechnology, Santa Cruz, CA, USA), antitumor necrosis factor-alpha (TNF-α, 1:200, Catalog No. 654250, Calbiochem, San Diego, CA, USA), antihuman CD34 class II (CD34, 1:10, Catalog No. MCA547T, NC), antihuman substance P (1:200, Catalog No. sc-9758, Santa Cruz Biotechnology), and antineuron-specific class III beta-tubulin (TUJ1, Catalog No. MAB1195, R&D Systems, Minneapolis, MN, USA). The sections were then incubated with peroxidase-labeled streptavidin–biotin (Histofine, Nichirei, Tokyo, Japan). Localization of the antigens was visualized using 3,3′-diaminobenzidine tetrahydrochloride dehydrate (DAB). Sections were washed, dehydrated, and mounted under coverslips using Permount (Fisher Scientific Chemical Division, Fair Lawn, NJ, USA). Five serial slides from the center of the weight-bearing area of the condyles were evaluated to assess the corresponding antigens. A specimen that had immunoreactivity in any of the slides was considered a positive specimen. A specimen that had no immunoreactivity on five slides was considered a negative specimen.

In addition to the immunohistochemical examinations, tartrate-resistant acid phosphatase (TRAP) staining was performed to detect osteoclasts.

Histological evaluation

Following H&E staining, the numbers of cystic lesions that evaded the subchondral bone plate or calcified zone were counted in 10-mm-long sections to determine the density of

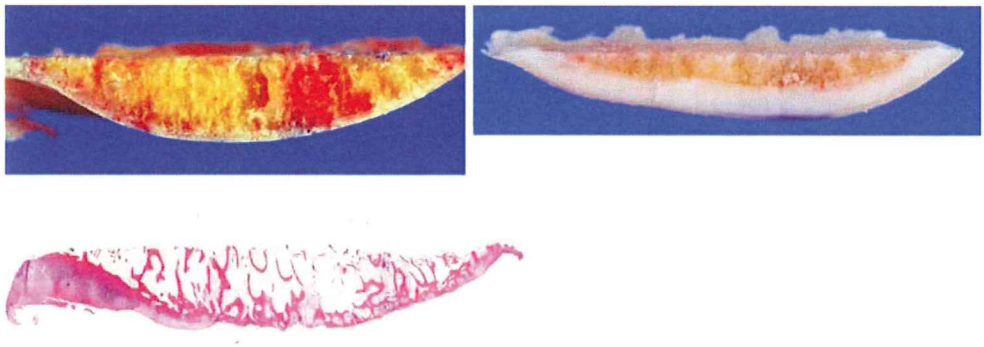

Fig. 1 Specimen obtained at the time of TKA. The *upper left* and *upper right* photos show representative examples of the MFC and LFC, respectively, which were obtained by a distal bone cut at the time of TKA. The specimens were then cut in half sagittally. The *lower photo* shows typical H&E staining of the MFC. Note that the majority of the surface of the MFC was denuded and partially covered with fibrous tissue, whereas the cartilage was preserved on the surface of the LFC

cystic lesions [16]. In addition, the cell populations forming the cystic lesions were analyzed. For this analysis, CD34 immunoreactive cells were identified as endothelial cells, polynuclear cells were identified as osteoclasts, and other cells were identified as mononuclear cells. The number of cells that was found in a cyst was also counted.

Statistics

Statistical analysis was done using the Mann–Whitney *U* test. A p value <0.01 was considered statistically significant.

Results

Patients

Of the 15 subjects examined, two were male and 13 were female, ranging in age from 62 to 79 years old (mean 67.7 years old). X-ray images of the medial compartment were grade IV on the K/L scale for all of the patients, whereas three lateral compartments were grade I and 12 were grade II. BME was detected in the MFC and the medial tibial plateau. No BME was detected in the LFC, as determined in the inclusion criteria. These results implied minimal, if any, arthritic changes in the lateral compartment. Thus, all of the patients in this series were considered to have medial-type OA.

Histological evaluation

H&E staining revealed that, in all of the cases, the articular cartilage in the weight-bearing portion of the MFC was worn and part of the surface was covered with fibrous tissue (Fig. 1). Representative H&E staining of the subchondral bone of the MFC and the LFC is shown in Fig. 2. Cystic lesions consisting of fibrous tissue were found in the subchondral plate, both in the MFC and in the LFC. The number of cystic lesions was 2.2±0.10/10 mm in the MFC and 0.47±0.22/10 mm in the LFC. The average cell number for each cyst was 94.6±34.2 in the MFC and 55.2±24.0 in the LFC. The frequency of cystic lesions was significantly higher in the MFCs than the LFCs. TRAP staining to identify osteoclasts and antibodies against CD34 to identify vascular endothelial cells clearly showed the existence of both cells (Fig. 3). The proportion of mononuclear cells in a cystic lesion was 78.7% in the MFC and 77.2% in the LFC. The proportions of osteoclasts and endothelial cells in a cystic lesion were 1.2% and 21.2% in the MFC and 0.6% and 22.4% in the LFC, respectively. No significant difference in the types of cells in the cystic lesions was found between the MFC and the LFC (Table 1).

Immunohistochemical examination revealed that certain cells or interstitial tissue in the cystic lesions in the subchondral bone plate in the MFC stained positive for Cox-2 (Fig. 4g, k), TNF-α (Fig. 4h, l), TUJ1 (Fig. 4i, m), and substance P (Fig. 4j, n). The proportion of antibody-positive specimens was as follows: Cox-1, four out of 15 cases; Cox-2, 15 out of 15 cases; and TNF-α, 13 out of 15 cases. Substance P-positive and TUJ1-positive fibers were found in the MFC (15 out of 15). No antibody-positive specimens or fibers were found in the LFC (Table 2). As for nerves in the Haversian canal, TUJ1-positive fibers were detected in both the LFC and MFC. Certain cells in the synovium also stained positively for Cox-1, Cox-2, and TNF-α. The proportion of antibody-positive synovial specimens was as follows: Cox-1, one out of 15 cases; Cox-2, 15 out of 15 cases; TNF-α, 12 out of 15 cases; substance P, 15 out of 15 cases; and TUJ1, 15 out of 15 cases (Table 2).

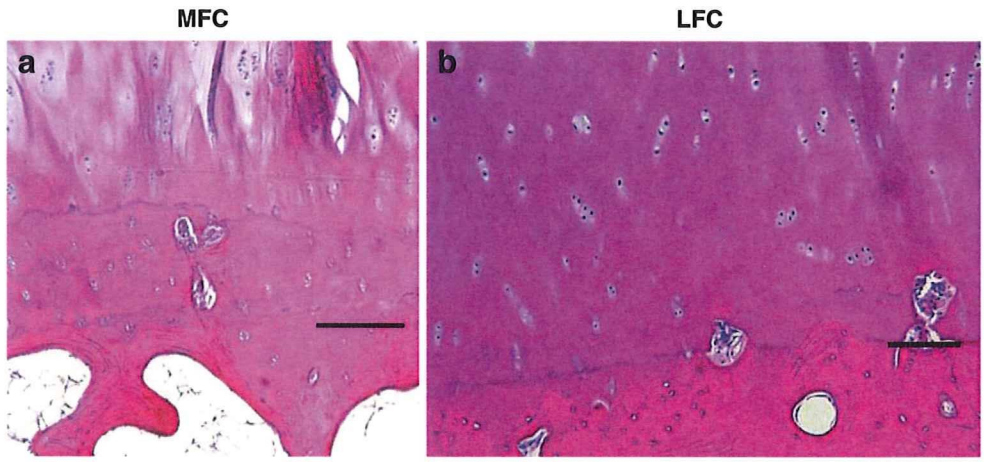

Fig. 2 Cystic lesion in the MFC. The *left photo* (**a**) shows a typical cystic lesion at the boundary of the bone and cartilage in the MFC. The lesions were also found in the LFC (*right photo*, **b**). In this specimen, the surface of the cartilage was fibrillated in the MFC and cell cloning was observed in the LFC

Discussion

Relationship between knee pain and TUJ1, substance P, Cox-2, and TNF-α

In this study, we selected medial-type OA patients with minimal involvement of the lateral compartment based on X-ray and MRI examinations and found exclusive expression of TUJ1, substance P, Cox-2, and TNF-α in the MFC but not in the LFC. By setting the criteria such that those with BME in the lateral compartment were excluded, comparisons between affected compartments and minimally affected or unaffected compartments were possible since even low-grade X-ray examination of the knee will detect BME as an indication of early osteoarthritic changes [17]. The detection of substance P, Cox-2, TNF-α, and TUJ1 indicated that pathological changes in the subchondral plate that occurred in the affected knee compartment can be a source of knee pain, although the main source of these molecules has been thought to be the synovium [18–21]. Although the biological activities of substance P, Cox-2, TNF-α are pleiotropic, and simple detection of their existence does not necessarily indicate pain provocation, they are still considered to be pain-related or inflammatory molecules [12, 18, 19, 22–24].

Substance P is a neurotransmitter that causes reflex neurogenic inflammation in the joint after being released from afferent nerve fibers [25, 26]. Administration of substance P into the knee joint has been reported to increase the severity of arthritis in an experimental rat model [25]. Cox-2 is rapidly induced in instances of tissue injury and inflammation [27]. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) to inhibit Cox-2 activity is one of the most common treatments for OA knee pain, and the relatively high efficacy of NSAIDs in relieving knee pain suggests the involvement of Cox-2 in the generation of knee pain [28, 29]. TNF-α has been reported to play a pivotal role in the development of inflammatory hyperalgesia [23], and downregulation of TNF-α has been reported to be one of the mechanisms for pain reduction obtained by high-molecular-weight hyaluronan [30]. Thus, substance P, Cox-2, and TNF-α are among the key molecules involved in OA knee pain.

Although TUJ1 is a pan-neuronal marker and does not differentiate sensory neurons from other neurons, the exclusive, positive immunoreactivity of TUJ1 in the subchondral bone of the MFC implies the occurrence of nerve ingrowth only in the MFC. The relatively immediate reduction in pain obtained by TKA might account for the involvement of the subchondral bone in knee pain because

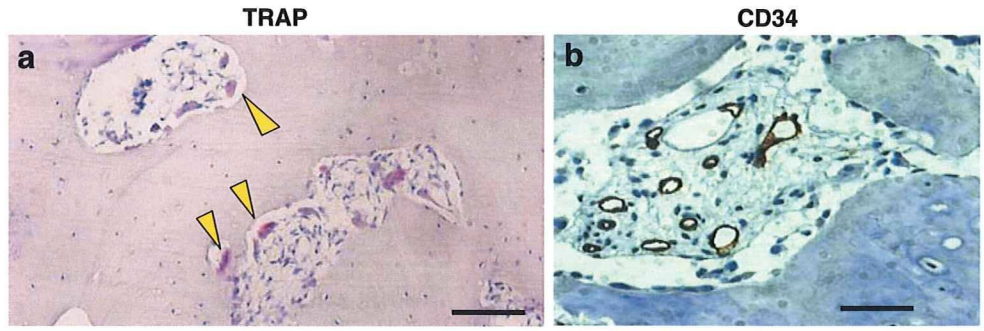

Fig. 3 Cells in a cystic lesion. Multinucleated, TRAP-positive cells were considered to be osteoclasts (*left*, *arrows*, **a**), and CD34-positive cells were regarded as vascular endothelial cells (*right*, **b**)

Table 1 Cell constituents of cystic lesions (in percent)

	Mononuclear cells	Osteoclasts	Vascular endothelial cells
MFC	78.7±8.36	1.20±1.21	21.2±8.33
LFC	77.2±8.00	0.6±1.10	22.4±7.79

MFC medial femoral condyle, *LFC* lateral femoral condyle

most of the affected subchondral plate is excised in TKA (debridement effect of TKA).

A discrepancy was found between X-ray image findings and pain severity in OA knee patients during daily, outpatient clinic evaluations. The X-ray image findings showed mild OA, but the patients' gonalgia was severe. This result might be accounted for by the expression levels of Cox-2, TNF-α, substance P, and nerve ingrowth, although in this study, we only examined cases that were painful enough to require TKA.

Cystic lesions

Positive immunoreactivities of TUJ1, substance P, Cox-2, and TNF-α were detected in cystic lesions that formed in the subchondral plate of the MFC. These cystic lesions have been called vascular channels, subchondral cracks, or bone resorption pits and are reported to be the result of an invasion from the bone marrow [12, 16, 31, 32].

Shibakawa et al. reported that the density of grade II bone resorption pits, which they defined as bone marrow tissue that infiltrated beyond the tidemark, was high in the medial tibial plateau in the medial-type OA knee [16]. This appeared to be consistent with our results showing that the density of cystic

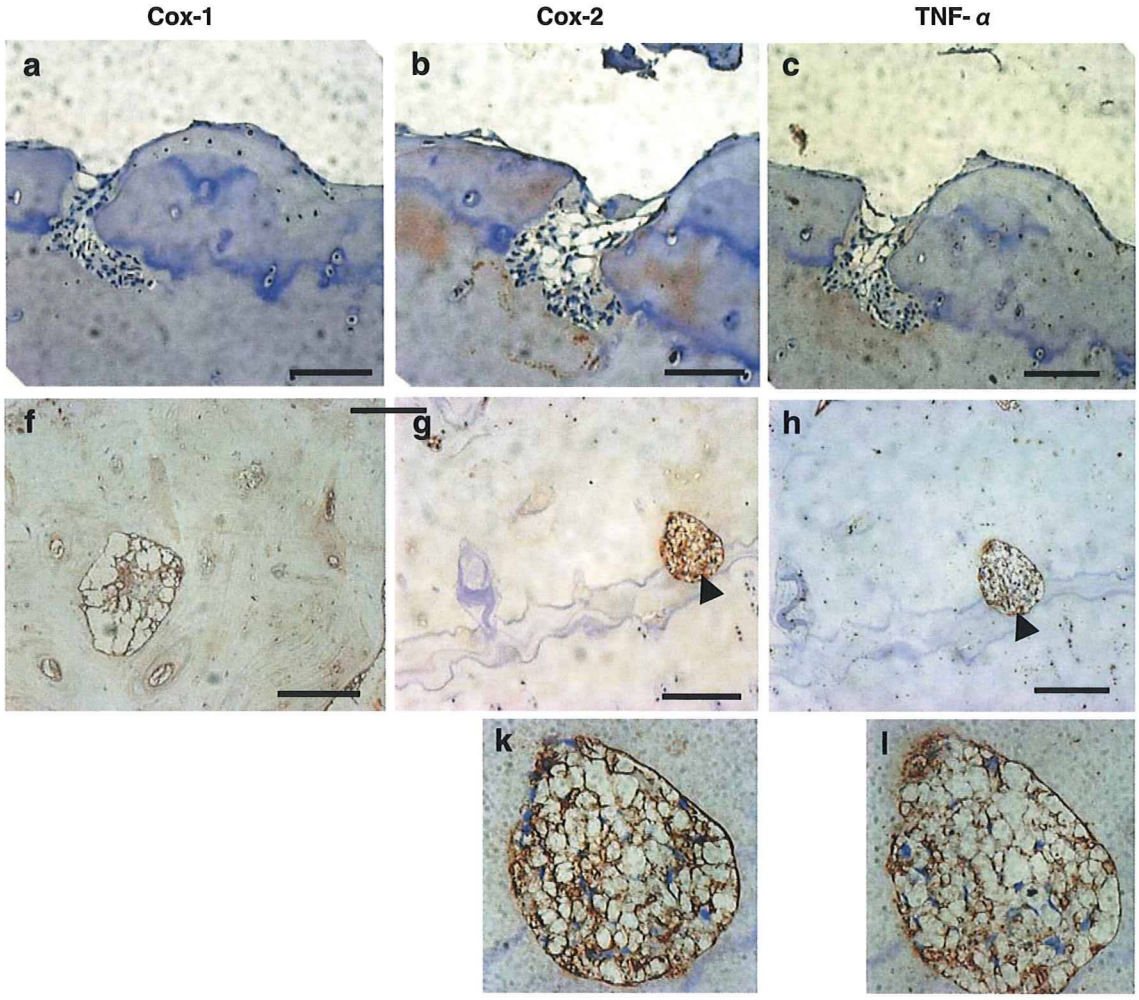

Fig. 4 Immunohistochemical analysis of subchondral bone from an osteoarthritic knee. The MFC and the LFC were stained with antibodies raised against Cox-1, Cox-2, TNF-α, substance P, and TUJ1. *Upper lanes* (**a**–**e**) show specimens from the LFC, and *middle lanes* are from the MFC (**f**–**j**). Cystic lesions in the MFC contained immunopositive cells or fibers (indicated by *arrowheads*), but those in the LFC did not. Scale bar denotes 100 μm. Higher magnification of cystic lesions were presented at the *lower lanes* (**k**–**n**). Cox-2, TNF-α, TUJ1, and substance P were all immunopositive in the cytoplasm as well as interstitial tissue but types of cells producing these molecules could not be identified

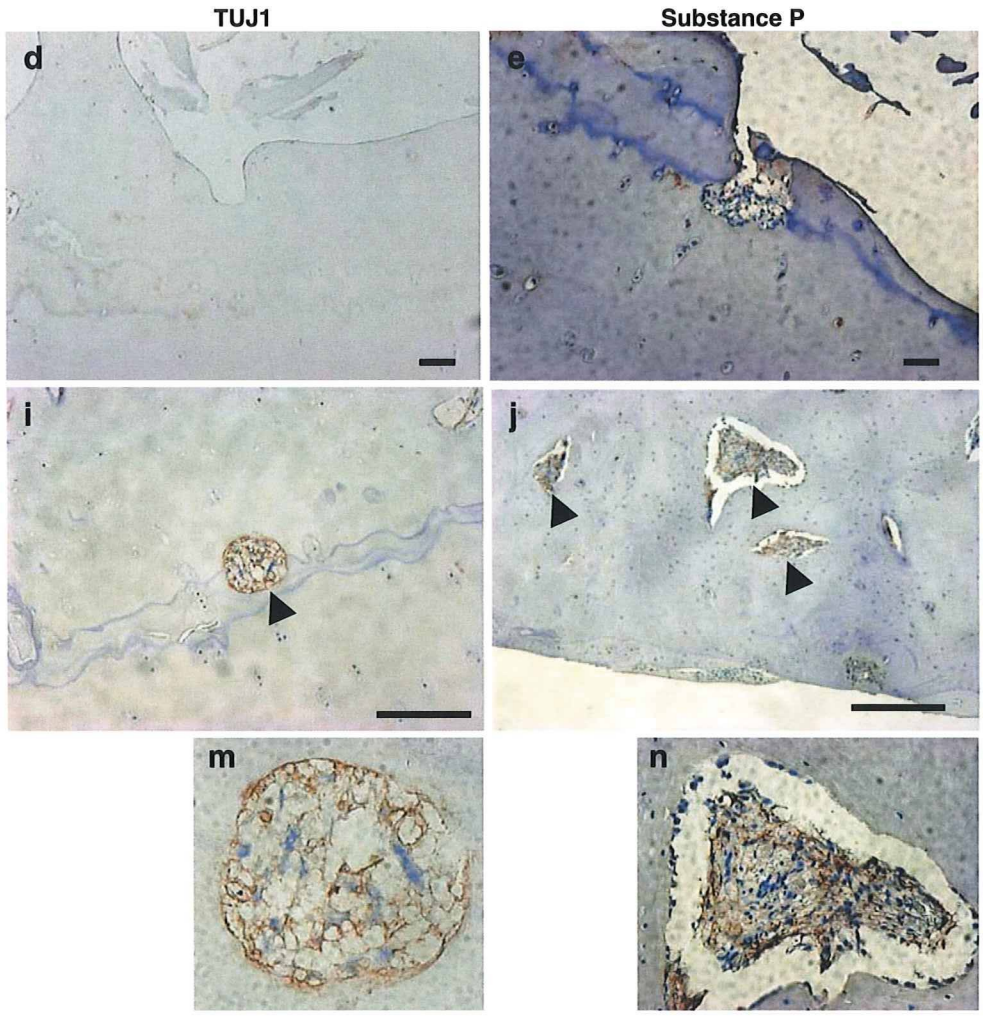

Fig. 4 (continued)

lesions was high in the MFC, but we did not distinguish them by their location because even when they were located within calcified cartilage as seen on a slide, they might have gone beyond the tidemark at other articular surfaces.

In this study, immunohistochemical analysis indicated that the cystic lesions were phenotypically different between the MFC and the LFC. Mononuclear cells, polynuclear cells, and vascular endothelial cells were all observed between the MFC and LFC and there was no difference regarding the cell constituents at the microscopic level. TRAP-positive polynuclear cells were considered to be osteoclasts (Fig. 3). Cells forming vessels were recognized as vascular endothelial cells because of their CD34-positive character (Fig. 3). Fibroblastic mononuclear cells were considered osteoblasts or preosteoblasts based on their morphology. Cells adjacent to bone tissue appeared as osteoblasts and cells farther away from the bone tissue appeared as preosteoblasts or stromal cells. However, they might have been attached to bone prior to the staining process. Alkaline phosphatase staining would be appropriate for differentiating osteoblasts, but this was difficult in the present study due to the use of decalcified specimens. Several stimuli are known to induce Cox-2 expression as well as TNF-α expression in osteoblasts or preosteoblasts [33–38]. Cox-2-positive or TNF-α-positive mononuclear cells in the cystic lesion did not contradict the notion that these were osteoblasts or preosteoblasts. Thus, osteoblasts or preosteoblasts, osteoclasts, and endothelial cells appeared to be the main constituents of the cystic lesions. Unidentified, specific cells may exist, but we can only state that cells in the MFC and LFC are morphologically the same even though they are phenotypically different.

Table 2 Proportion of positive specimens

	Cox-1	Cox-2	TNF-α	Substance P	TUJ1
MFC	14/15	15/15	13/15	15/15	15/15
LFC	0/15	0/15	0/15	0/15	0/15
Synovium	1/15	15/15	12/15	15/15	15/15