

Histological and biological comparisons between complete and incomplete discoid lateral meniscus

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Abstract

The discoid lateral meniscus (DLM) is an anatomically abnormal meniscus that covers a greater area of the tibial plateau than the normal meniscus. The DLM is classified into 2 types: complete (CDLM) and incomplete (ICDLM) types. In this study, we investigated the histological and cell biological characteristics of CDLM and ICDLM. The number of blood vessels, proteoglycan deposition, and collagen distribution were assessed using meniscal tissues. Collagen production was also investigated in CDLM and ICDLM cells. The intercondylar region of the CDLM had higher number of blood vessels than the inner region of the ICDLM. Safranin O staining density and type II collagen deposition in ICDLM were higher than those in CDLM. Type II collagen-positive cells were higher in ICDLM than in CDLM. CDLM cells showed slender fibroblastic morphology, while ICDLM cells were triangular chondrocytic in shape. This study demonstrated that the intercondylar region of the CDLM showed similar properties to the outer region of the meniscus. The inner region of the ICDLM, on the other hand, differs from the intercondylar region of the CDLM. Our results suggest that the intercondylar region of the CDLM may have a high healing potential like the outer meniscus.

Keywords: Discoid lateral meniscus, blood vessel, proteoglycan, type II collagen, Cellular morphology

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Introduction

Discoid meniscus is a morphological abnormality where the meniscus covers the tibial plateau in an abnormally wide and thick layer. First reported in 1889 by Young [1], discoid meniscus mainly appears in the lateral meniscus (LM) [2]. Its incidence varies, ranging from 0.4% to 17%: high incidence has been reported in East Asian countries like Japan and Korea, but the reason for this is unclear [3-5]. Araki et al. defined discoid meniscus as when the meniscus is found to have a transverse cross-section of ≥ 14 mm in magnetic resonance imaging studies [6]. The Watanabe classification scheme is used to classify discoid lateral meniscus (DLM) into complete DLM (CDLM), defined as when the meniscus completely covers the lateral tibial plateau and incomplete DLM (ICDLM), as when the meniscus does not completely cover the tibial plateau. Additionally, Wrisberg-ligament type DLM occurs when the meniscus covering the tibial plateau is attached to only the posterior menisiofemoral ligament [7]. The Wrisberg-ligament type is now considered to be a congenital defect of the posterior coronary ligament and is believed to present with hypermobility and occasionally cause subluxation, because the lateral meniscus (LM) is supported only by the ligament of Wrisberg [8].

Located within the knee joint, the meniscus contributes to load distribution, shock absorption, joint stabilization, and joint lubrication. The meniscus functions to distribute the burden placed on the knee-joint cartilage by axial load in the form of hoop stress, by flexing outwards [2].

Biologically, the anatomically normal meniscus in terms of wet weight is comprised of 72% water and the remaining 28% is made up of extracellular matrix and cells. Collagen comprises 75% of these organic compounds, of which 98% is type I collagen, whereas type II collagen fibers are concentrated at the surface layer [9, 10]. However, no previous studies have examined the structural components and cytological characteristics of DLM. Further, DLM is known to have a higher risk of meniscal injury compared with normal meniscus [11]. While asymptomatic DLM does not require treatment, the appearance of symptoms as a result of meniscal injury such as pain, clicking, and limited extension necessitates surgical treatment. In recent years, with the objective of preserving meniscal function, saucerization is performed at first. Subsequently, in cases where a tear remains or instability is observed, meniscal repair is provided. Outcomes of this protocol are generally favorable [12, 13, 14]. However, whereas partial meniscectomy results in remission of symptoms in many cases of ICDLM, the tear spans a wide area in CDLM. Therefore, the accompanying partial loss of the outer meniscal region often hinders treatment.

According to a report by Cui et al. [15], CDLM can be divided into 7 layers based on the arrangement of collagen fibers, where the central layer can be divided into a medial middle zone having an irregular fiber arrangement and a lateral middle zone having a circular fiber arrangement. However, there are no reports to date that have examined fiber organization in ICDLM. Reports are available, on the other hand, regarding the differences between meniscal injuries in CDLM and

ICDLM. Bin et al. reported that simple horizontal tears are found only in CDLM, whereas radial, degenerative, and complex tears are found only in ICDLM [16]. Moreover, the ages at which symptoms of meniscal injury are seen in CDLM and ICDLM are different. CDLM often requires surgical treatment in childhood, whereas ICDLM typically requires surgical treatment in adults. Based on these findings, we believe that DLM classification into CDLM and ICDLM reflects differences not only in morphology, but also in structural components and fiber orientations of the meniscus. Moreover, the biological characteristics of the meniscus cells are also different. We further believe that these differences likely underlie differences in tear morphology and the age at which the meniscal injury is sustained. However, these characteristics in CDLM and ICDLM are still unknown. In this study, we investigated histological and cellular biological characteristics in both CDLM and ICDLM to understand the differences in their clinical features.

Materials and Methods

Meniscal sample preparation: Institutional Review Board approval and informed consent were obtained before all experimental studies. This study was performed according to the protocol approved by the Institutional Review Board of our institution. We obtained meniscal samples from the intercondylar region of CDLM and the inner region of ICDLM (Fig. 1). The intercondylar region of CDLM was obtained during arthroscopic partial meniscectomy. The intercondylar region

was defined as the inner 5-mm region of CDLM without meniscal roots. The inner 5-mm region of ICDLM was prepared by arthroscopic meniscectomy in patients suffering from lateral meniscal injuries. Six CDLM patients were 8, 9, 12, 12, 16, and 17 years of age. Six patients suffering from ICDLM were 16, 20, 20, 23, 24, and 31 years of age (Table, Supplemental Fig.).

Cells and cell culture: Meniscal samples derived from CDLM and ICDLM were minced. Each cell suspension was treated with collagenase (Sigma, St. Louis, MO, USA) as previously described [17, 18, 19]. Attached cells (passage 0) were maintained in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (HyClone, South Logan, UT, USA) and 1% penicillin/streptomycin (Sigma). DLM cells between passages 1 and 3 were used.

Blood vessel count: DLM samples were fixed in 10% formalin solution and embedded in paraffin blocks. Coronal sections of DLM samples (6- μ m-thick) were stained by safranin O dye as described [20]. A total of 5 locations, measuring 500 μ m \times 400 μ m = 0.2 mm² were selected on each prepared slide and the number of blood vessels were counted. Luminal structures surrounded by endothelial cells having nuclei were counted as blood vessels, and each luminal structure observed was counted as one blood vessel. Average values were calculated for each of 6 CDLM samples and 6 ICDLM samples, measuring 1 mm² each, and compared. To determine inter-observer and intra-observer reliability, 3 independent observers performed all representative

experiments three times using the above method, and results were found to be similar.

Deposition of proteoglycans: To quantify the deposition of proteoglycans in meniscal samples, signal density of safranin O-stained areas were assessed. Images were analyzed by Image J version 1.31 software, as described [21, 22]. Five regions of interest (ROIs), measuring 200×200 pixels each, were created within each tissue sample. Average intensity was calculated for each image, and the mean and standard deviation were calculated for the intercondylar region of CDLM and for the inner region of ICDLM.

Deposition of collagens: The deposition of type I and II collagens in the intercondylar region of CDLM and the inner region of ICDLM were assessed by immunohistochemical analyses using rabbit anti-type I collagen monoclonal antibody (Abcam, Cambridge, UK) and mouse anti-type II collagen monoclonal antibody (Kyowa Pharma Chemical, Toyama, JAPAN) antibody as described [23]. To quantify signal density, images were analyzed by Image J version 1.31 software, as described [21, 22]. The mean value derived from 5 different images was evaluated.

Cellular morphology and collagen synthesis: Cultured cells derived from CDLM and ICDLM were observed using a phase-contrast microscope. Immunohistochemical analyses were performed as

described [24]. Cells were fixed with 1% paraformaldehyde solution for 10 min, air-dried, blocked with 1% bovine serum albumin (BSA) for 10 min at room temperature. Slides were incubated with a rabbit anti-type I collagen monoclonal antibody and mouse anti-type II collagen monoclonal antibody for 1 h. BSA solution without the primary antibody was used as a negative control. Alexa Fluor 488-conjugated antibody (Invitrogen, Carlsbad, CA, USA), Alexa-Fluor 568-conjugated phalloidin (Molecular Probes, Eugene, OR, USA), and Hoechst 33342 (ICN Biomedicals, Aurora, OH, USA) were used for detection. Samples were examined under a fluorescence microscope (Leica, Wetzlar, Germany). Type II-positive cell percentage was measured as the ratio of cells positively stained with anti-type II collagen antibodies to total cell count over an area of $500 \mu\text{m} \times 400 \mu\text{m}$. A total of 6 CDLM and 6 ICDLM cultures were measured three times each, and the mean value was calculated.

Cell proliferation assay: Cell proliferation assays were performed as described [25]. In brief, DLM cells in microplates were incubated for 24 h in a final volume of $100 \mu\text{l}$ /well culture medium and incubated for 0, 24, 48, and 72 h prior to addition of the cell proliferation reagent, water-soluble tetrazolium-1 (Roche Diagnostics, Basel, Switzerland). The optical density was measured using a microplate reader at evaluation and control wavelengths of 450 and 650 nm, respectively. Data obtained by subtracting readings at 630 nm from those at 450 nm were used for evaluation ($n = 6$).

Statistical analysis: All experiments were repeated at least three times independently, and similar results were obtained. Data were expressed as mean \pm standard deviation. Differences among groups were compared using the Mann–Whitney U-test. Statistical significance was established at $p < 0.05$.

Results

The intercondylar region of the CDLM includes blood vessel formation: Blood vessels were observed in the intercondylar region of CDLM at a concentration of approximately 16.3/mm², but none were observed in the inner region of ICDLM. Likewise, a greater number of meniscus cells were observed in the intercondylar region of CDLM compared with the inner region of ICDLM (Fig. 2A-B). Fibers were organized in parallel in the intercondylar region of CDLM, but at random in the inner region of ICDLM. Blood vessel counts were significantly higher in the intercondylar region of CDLM than in the inner region of ICDLM (Fig. 2C).

The inner region of the ICDLM contains larger amounts of safranin O-stained proteoglycans: Safranin O staining was weaker in the intercondylar region of CDLM than in the inner region of ICDLM. Fiber arrangement was regular and meniscus cells were present along the fiber

arrangements in the CDLM (Fig. 3A). The inner region of ICDLM was stained red by safranin O dye. The fiber arrangement of the inner region of ICDLM was random, with meniscus cells forming sparse cell populations consisting of a few cells (Fig. 3B). In image analysis, staining density of ICDLM was approximately twice that of CDLM. Deposition of proteoglycans stained by safranin O was significantly higher in ICDLM (Fig. 3C).

The inner region of the ICDLM contains a larger quantity of type II collagen compared with the intercondylar region of the CDLM: Deposition of type I collagen was comparable for both the intercondylar region of CDLM and the inner region of ICDLM. Type I collagen was uniformly stained in the intercondylar region of CDLM, but some patchiness was observed in the inner region of ICDLM (Fig. 4A-B). No significant difference in type I collagen staining density was observed between groups (Fig. 4C). Intense staining was obtained for type II collagen in the inner region of ICDLM, but the intercondylar region of CDLM was almost unstained (Fig. 4D-E). In image analysis, relative type II collagen density was significantly higher in the inner region of ICDLM (Fig. 4F).

Cells derived from ICDLM inner region show chondrocytic morphology compared with fibroblastic cells derived from the intercondylar region of the CDLM: Distinctive cell

morphologies were observed using phase-contrast microscopy. Cell morphologies in CDLM were slender and fibroblastic, while ICDLM was characterized by triangular chondrocytic cell morphologies (Fig. 5A-B).

Type II collagen synthesis is higher in the inner cells of the ICDLM: Immunostaining revealed type I collagen production in both the CDLM and ICDLM cells (Fig. 5C-D). While type II collagen synthesis was observed in approximately 77% cells in ICDLM, approximately 10% CDLM cells were stained, constituting a significant difference (Fig. 5E-G).

Cells derived from CDLM intercondylar and ICDLM inner regions show similar proliferating activities: CDLM cells proliferated about 1.9-fold at 48 h and 2.2-fold at 72 h of culture (Fig. 6A). ICDLM cells proliferated approximately 1.5- and 2.0-fold at 48 and 72 h after culturing, respectively (Fig. 6B).

Discussion

Several reports are available regarding the vasculature of the meniscus. During the fetal period, the cellular component of the meniscus is high and richly innervated vasculature is observed across the entire region; however, this is observed in only the lateral third of the outer region in

adults [26]. The inner region of the meniscus, in contrast, is considered an avascular area [27]. Blood vessels were not observed in the inner region of ICDLM in the present study, suggesting avascular tissue similar to the inner region of anatomically normal meniscus. The intercondylar region of CDLM that had vascularity may differ in nature from the inner region of ICDLM. Fiber arrangement in the normal meniscus is circumferential in the central main layer, but radial in the lateral lamellar layer, and random in the medial lamellar layer [28]. On the other hand, CDLM shows irregular fiber organization in the center region and in the medial middle zone, including the intercondylar region, of the central layer [15]. In the present study, fiber arrangement in the inner region of ICDLM was random, the same as in the inner region of normal meniscus. However, the fiber arrangement in the intercondylar region of CDLM was organized (Fig. 2A-B, 3A-B). Based on these findings, we consider that the intercondylar region of CDLM is similar in nature to the outer region and that its characteristics differ from the inner region of the normal meniscus.

The meniscus includes many types of cells with different morphologies and different traits, and cells derived from the inner meniscus have the phenotype of fibrochondrocytes [29]. Safranin O-staining density was higher in the inner region of the ICDLM compared with that in the intercondylar region of CDLM, revealing that the inner region of the ICDLM contains many fibrochondrocytic cells and that it possesses characteristics similar to the inner region of the normal meniscus (Fig. 3C). The present study showed that, although type I collagen is expressed in the

intercondylar region of CDLM as well as in the inner region of ICDLM, type II collagen is distinctively expressed in the inner region of ICDLM, when compared to its levels in the intercondylar region of CDLM (Fig. 4C, F). Similar results were obtained in cultured DLM cells (Fig. 5C-F), suggesting that the intercondylar region of CDLM and the inner region of ICDLM have different collagen-related properties. We have previously reported that, in the normal meniscus, type I collagen gene is expressed in both, inner and outer meniscus cells, but type II collagen gene expression is characteristic of inner meniscus cells [17, 18, 30]. According to reports by Moyer et al. and Fithian et al., by dry weight, type I collagen constitutes 80% of the red-red zone of the lateral third of the meniscus; collagen constitutes 70% of the medial third of the inner region (of which, 60% is type II collagen and 40% is type I) [9, 10]. In the present study, cells derived from the ICDLM inner region were characterized by type II collagen expression (Fig. 5G), indicating that their characteristics are similar to those of inner meniscus cells. The intercondylar region of CDLM, however, is characterized by its resemblance to normal outer meniscus cells, in terms of its cell morphologies and its extracellular matrix composition being primarily composed of type I collagen. We demonstrated that the intercondylar region of CDLM was characterized by slender cells, and the inner region of ICDLM by small round cells (Fig. 5A-D). Verdock et al. report that cells in the inner region of normal meniscus are fibrochondrocytic, small, and round in appearance, while fibroblastic and vascular endothelial cells are seen in the outer region [29, 31].

In this study, cells in the inner region of ICDLM exhibited a small, round shape, and were considered to be fibrochondrocytic cells, having a similar nature to those in the inner region of a normal meniscus. Cells in the intercondylar region of CDLM were found to have a phenotype similar to that of normal, outer meniscus cells.

In this study, differences in term of histological and biological characteristics were detected between the intercondylar region of CDLM and the inner region of the ICDLM. However, the mechanism underlying these differences remains unknown. We could not elucidate the properties underlying the differences between meniscal injuries in CDLM and ICDLM. More recently, reconstructive resection with suturing has been adopted as a treatment for DLM. Concurrent meniscal repair with saucerization has shown good clinical results in the treatment of DLM [12, 13]. While meniscal allografts and tissue-engineered meniscus can be used for the treatment of meniscal defect, their long-term clinical outcomes are poor [32, 33, 34]. We expect that a new treatment can be established by clarifying the mechanism underlying the differences observed between CDLM and ICDLM.

Limitations of this study include small sample size and large variations in age of the patients. Furthermore, the additional possibility that a phenomenon similar to angiofibroblastic hyperplasia caused by repetitive load may have occurred and that the number of blood vessels in the intercondylar region of the complete discoid meniscus was increased. Further investigation is

necessary to understand the properties of the intercondylar region of CDLM.

In conclusion, we demonstrated that the intercondylar region of CDLM shows similar properties of the outer region of a normal meniscus. The inner region of the ICDLM, on the other hand, differs from the intercondylar region of the CDLM, possessing characteristics similar to the inner region of a normal meniscus. With these findings, our results suggest that the intercondylar region of CDLM may have a high healing potential like the outer region of the meniscus.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure legends

Fig. 1. Meniscal tissue samples. (A-C) Intercondylar region of the CDLM. (B) Intercondylar region was resected. (C) Resected tissues of the intercondylar region. (D-F) Inner region of the ICDLM. (E) Partial meniscectomy was performed. (F) Resected tissues of the inner region. Bars, 5mm.

Fig. 2. Blood vessels in the DLM. (A) Intercondylar region of the CDLM. Arrows, blood vessels. (B) Inner region of the ICDLM. (C) Blood vessel counts in the CDLM and ICDLM. Bars, 100 μm .
* $p < 0.05$.

Fig. 3. Deposition of safranin O-stained proteoglycans in the DLM. (A) Intercondylar region of the CDLM. (B) Inner region of the ICDLM. (C) Relative safranin O staining density in the CDLM and ICDLM. Bars, 200 μm . * $p < 0.05$.

Fig. 4. Deposition of collagens in the DLM. Type I collagen deposition in the CDLM (A) and ICDLM (B). (C) Relative type I collagen density in the CDLM and ICDLM. Type II collagen deposition in the CDLM (D) and ICDLM (E). (F) Relative type II collagen density in the CDLM and ICDLM. Insets, negative controls. Bars, 200 μm . * $p < 0.05$.

Fig. 5. Cellular morphology and collagen deposition. Cell shape derived from the CDLM (A) and ICDLM (B). Type I collagen synthesis (red) in the cells derived from the CDLM (C) and ICDLM (D). Type II collagen deposition (green) in the cells derived from the CDLM (E) and ICDLM (F). (G) Type II collagen-positive cell percentage. Bars, 50 μm . * $p < 0.05$.

Fig. 6. Cellular proliferation. Relative cell proliferation ratios in the cells derived from the CDLM (A) and ICDLM (B). * $p < 0.05$.

Supplemental Fig. Arthroscopic findings of all CDLM cases. A 8-year-old man showed a horizontal tear of the CDLM (A, B). A 9-year-old man had no tear of the CDLM (C, D). A 12-year-old man had a horizontal tear of the CDLM (E, F). A 12-year-old man had a longitudinal tear of the CDLM (G, H). A 16-year-old man showed no tear of the CDLM (I, J). A 17-year-old woman had a longitudinal tear of the CDLM (K, L). All cases showed no tear in the intercondylar region of CDLM.

Table legend

Table. Demographics of the patients.

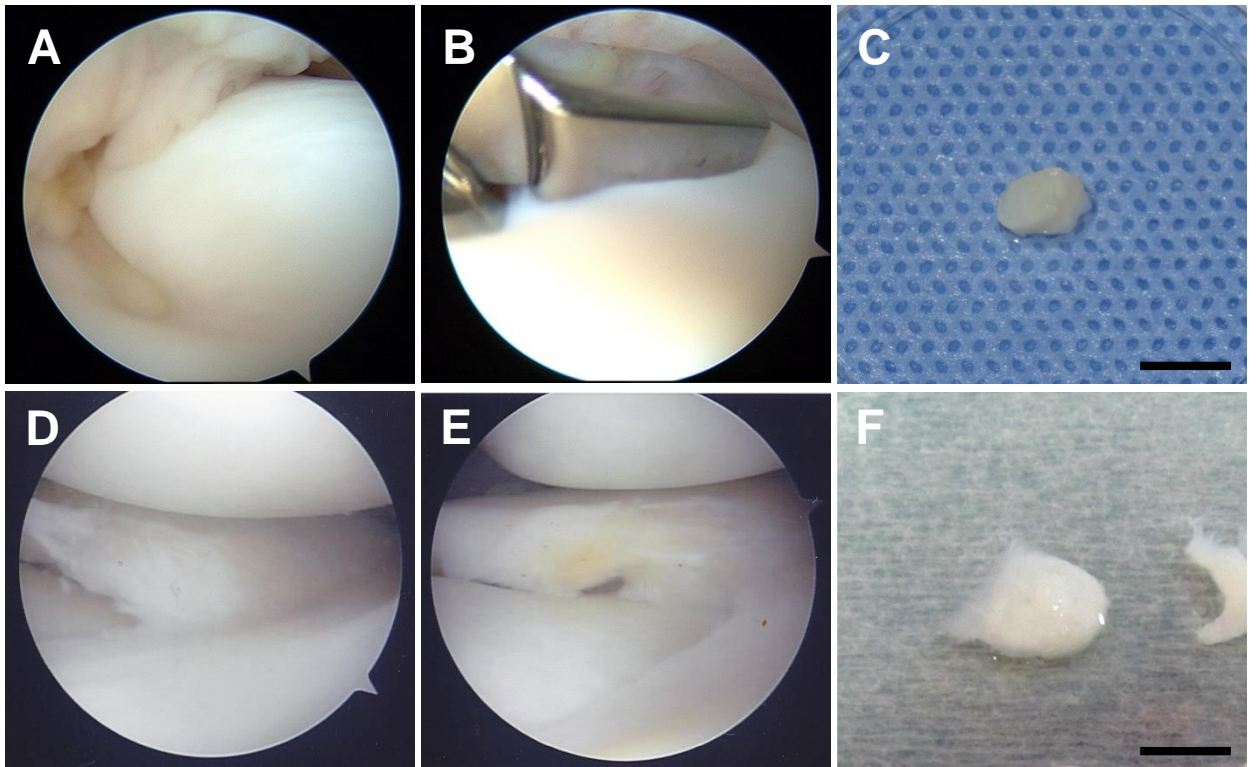


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Figure 1
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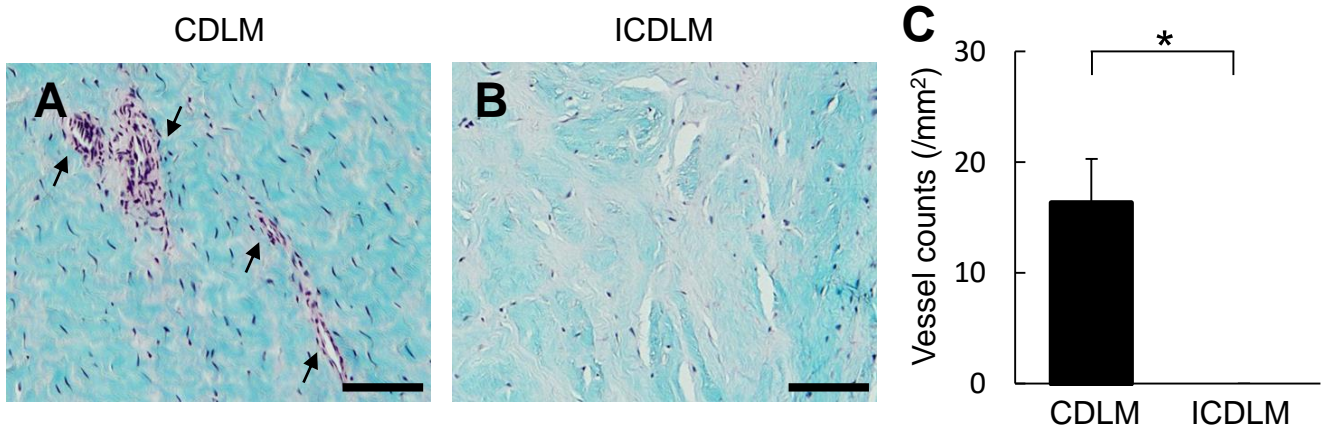


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Figure 2
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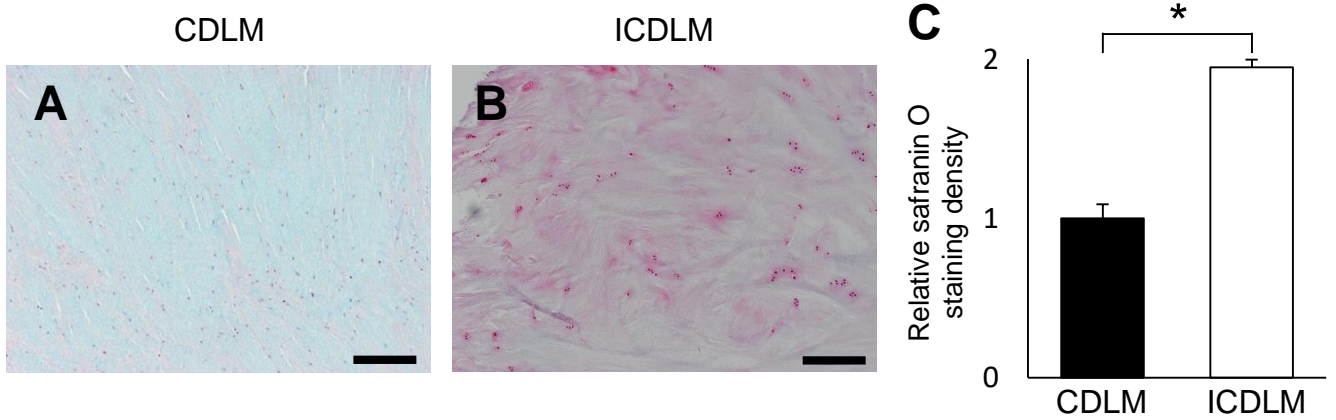


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Figure 3
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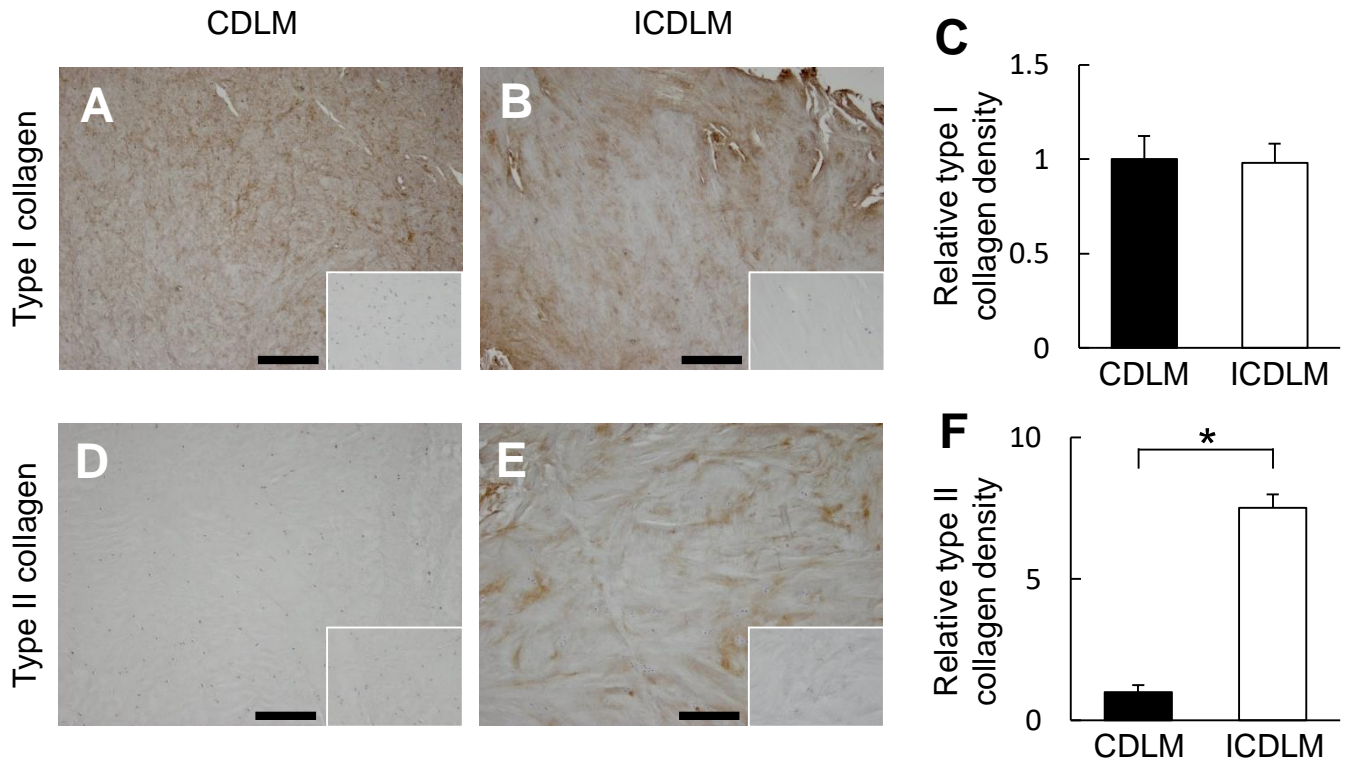


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Figure 4
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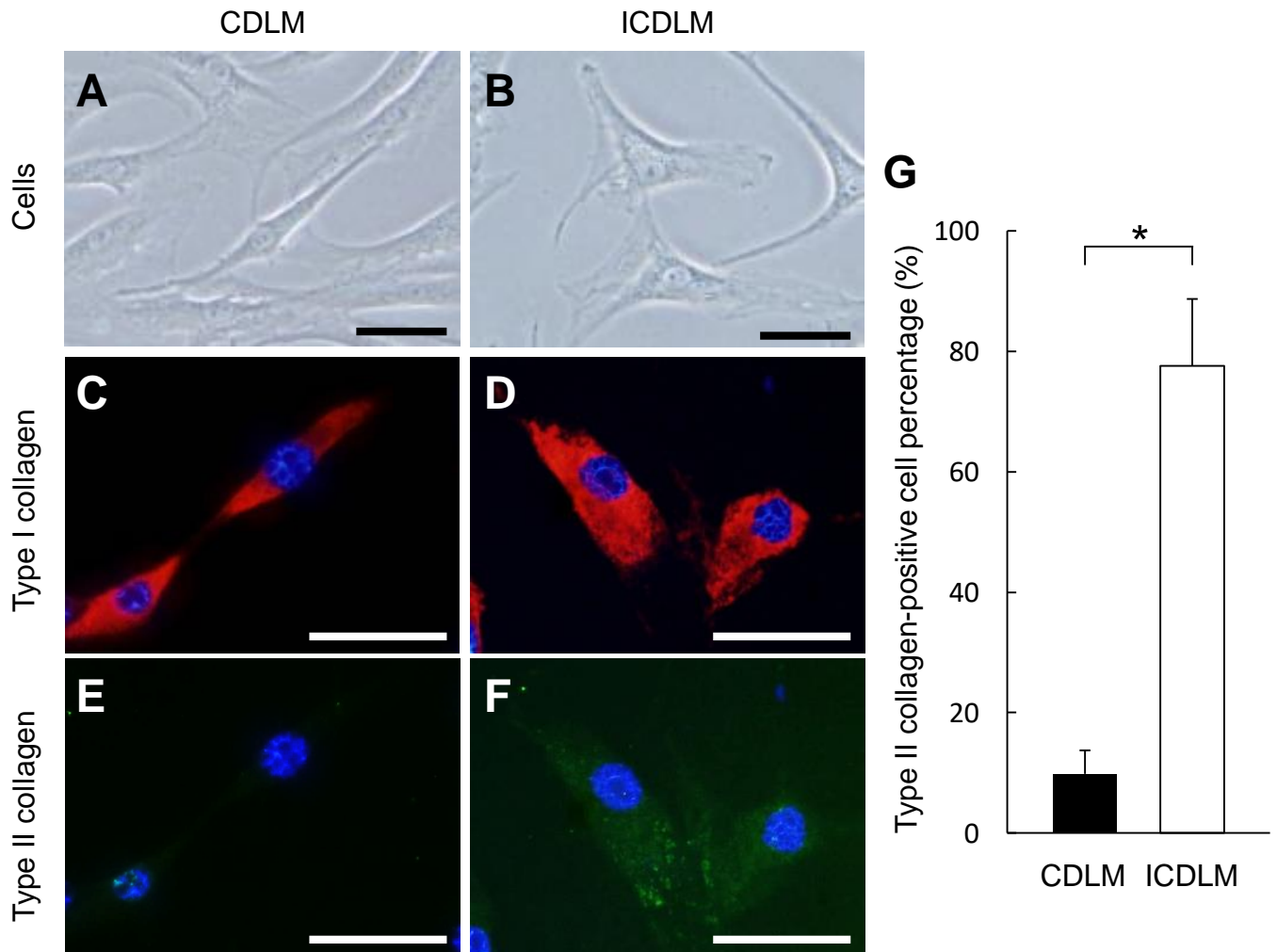


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Figure 5
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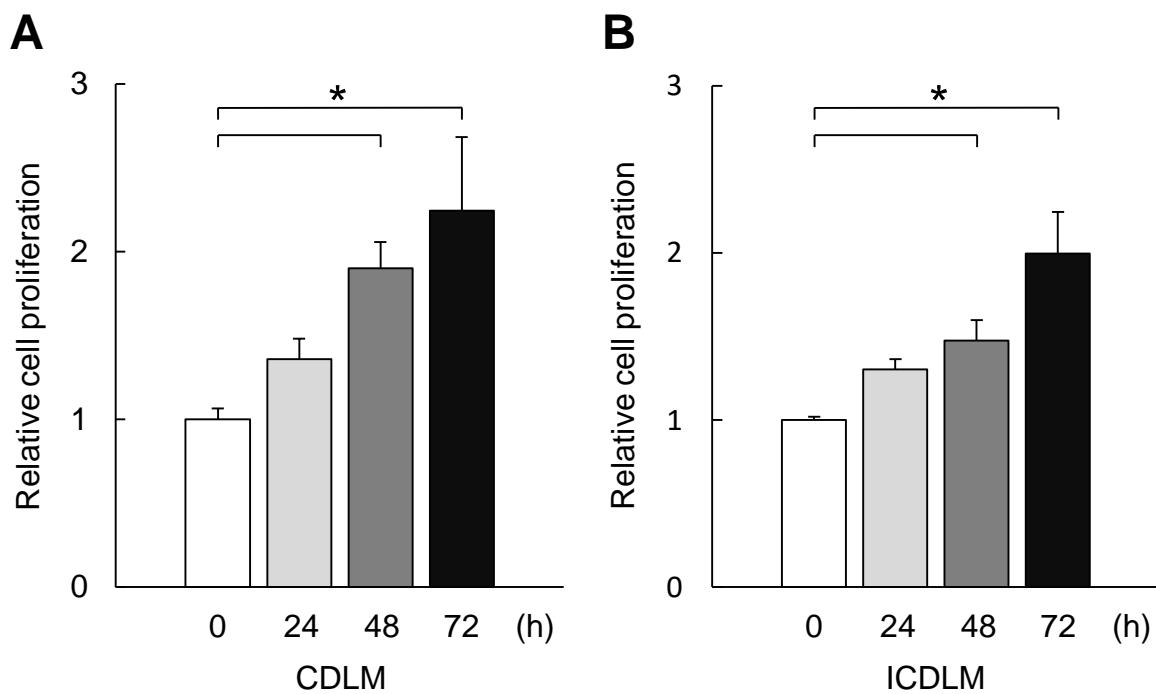


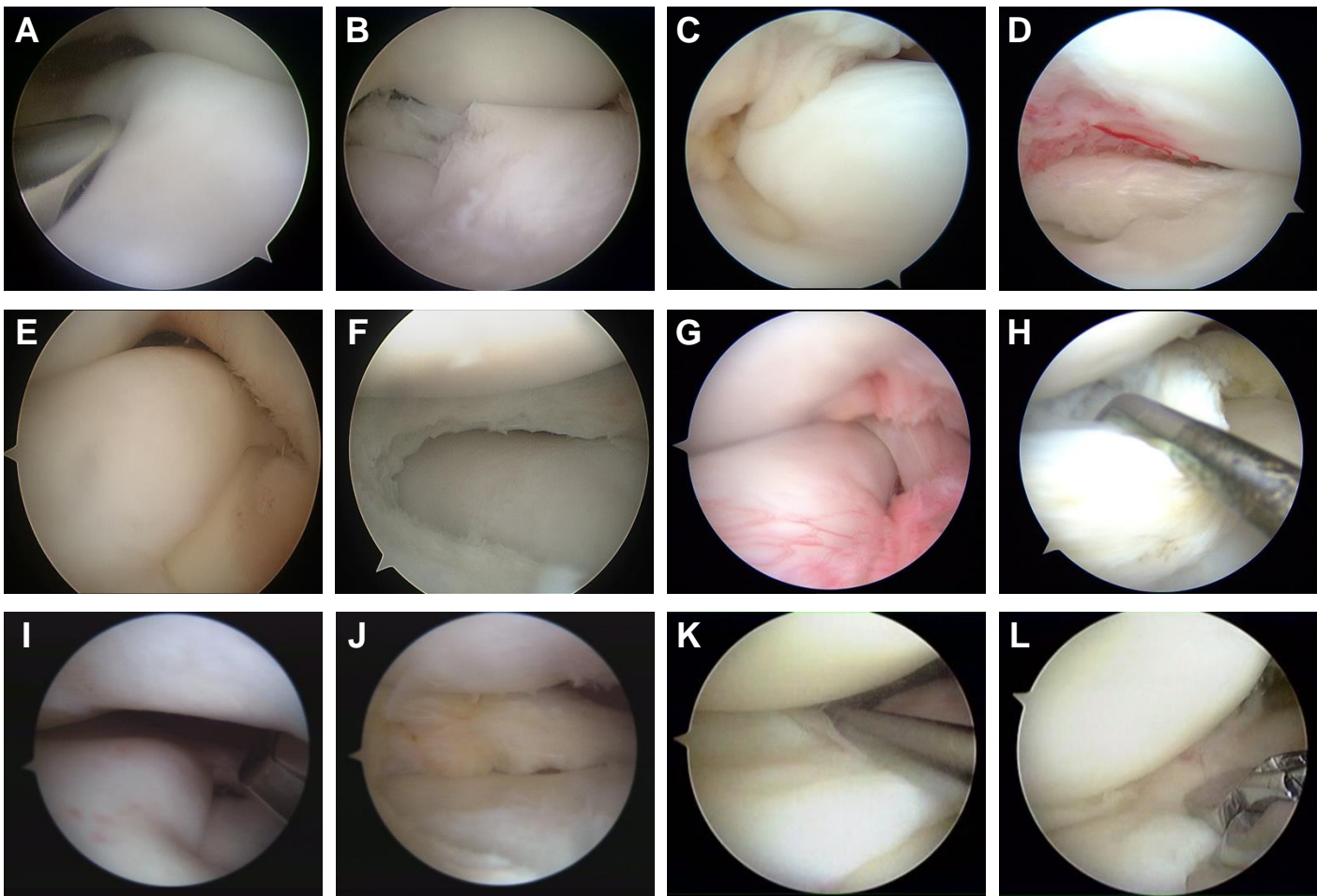
Fig. 6. Cellular proliferation. Relative cell proliferation ratios in the cells derived from the CDLM (A) and ICDLM (B). * $P < 0.05$.

Table. Demographics of the patients

Patient No.	Age (years)	DLM Type	Duration of Symptoms (months)	Tear Type	Operative Procedure
1	8	C	12	Horizontal	Partial + R
2	9	C	3	None	Partial
3	12	C	26	Horizontal	Partial + R
4	12	C	7	Longitudinal	Partial + R
5	16	C	8	None	Partial
6	17	C	10	Longitudinal	Partial
7	16	IC	4	Longitudinal	Partial + R
8	20	IC	3	Radial	Partial
9	20	IC	10	Horizontal	Partial + R
10	23	IC	7	Longitudinal	Partial + R
11	24	IC	12	Radial	Partial
12	31	IC	6	Degenerative	Partial

DLM, discoid lateral meniscus; C, complete; IC, incomplete; Partial, partial meniscectomy; R, meniscal repair.

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Supplemental Fig. Arthroscopic findings of all CDLM cases. A 8-year-old man showed a horizontal tear of the CDLM (A, B). A 9-year-old man had no tear of the CDLM (C, D). A 12-year-old man had a horizontal tear of the CDLM (E, F). A 12-year-old man had a longitudinal tear of the CDLM (G, H). A 16-year-old man showed no tear of the CDLM (I, J). A 17-year-old woman had a longitudinal tear of the CDLM (K, L). All cases showed no tear in the intercondylar region of CDLM.

Supplemental Figure
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