Angiogenic activity of subchondral bone during the progression of osteoarthritis in a rabbit anterior cruciate ligament transection model

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Objective: To investigate the longitudinal angiogenic activity of subchondral bone and cartilage during the progression of osteoarthritis (OA) using a rabbit model of OA.

Materials and methods: OA was surgically induced by anterior cruciate ligament transaction (ACLT) in left knee of 12 months old female New Zealand white rabbits (n = 33). Histological examination, immunohistochemistry, and angiogenic activity assay was done at 0, 2, 4, 6, 8, 12 weeks after ACLT. Histologic evaluation was performed with haematoxylin and eosin, safranin-O staining to assess the OA change of medial femoral condyle (MFC) and lateral femoral condyle (LFC). CD31 immunohistochemistry was performed to confirm the vascular invasion at osteochondral junction. A co-cultured tubule formation assay was conducted to evaluate angiogenic activity of the subchondral bone and cartilage of MFC and LFC as well as synovium. Association between histological changes, angiogenic activity, and vascular invasion were evaluated.

Results: OA changes increased in a time-dependent manner both in MFC and LFC. Angiogenic activity of subchondral bone showed a monomodal change during the OA progression, achieved a peak in the early to progressive stage and decreased to normal level in the late stage of OA. Surge of vascular invasion was observed following the increase of angiogenic activity in the progressive stage of OA. Angiogenic activity of cartilage did not change during the course of OA progression.

Conclusion: Angiogenic activity of subchondral bone was elevated in the early to progressive stage of OA and vascular invasion into the osteochondral junction followed.

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Introduction

Several factors associated with disease initiation and progression of osteoarthritis (OA) has been identified. One such factor is angiogenesis, a complex process through which new blood vessels develop from a pre-existing vasculature. Resultant invasion of vasculature into the osteochondral junction from subchondral bone has been reported to lead to cartilage degradation. Histological analysis has been used to study these features in human osteoarthritic knees. Elevated vascularity has been reported at the osteochondral junction of end-stage osteoarthritic knees compared to normal controls. In addition, elevated vascularity was observed in subchondral bone in the medial compartment compared to the lateral compartment in medial-type osteoarthritic knees, and elevated vascularization associated with innervation of sensory and sympathetic nerves into cartilage was detected at osteochondral junction in end-stage osteoarthritic knees. All of these results suggest a strong association between severity of OA and vascularity of the osteochondral junction; however, when the actual invasion occur remains to be elucidated. Vascular invasion might occur constantly during the progression of OA or it might surge in a specific phase of OA. A few animal studies implicated the early vascular invasion, but the longitudinal change of the vascular invasion at the osteochondral junction has been unknown.

In the present study, time-dependent change of angiogenic activity of OA knee was investigated. Angiogenesis are regulated by angiogenic activity of tissues, which is determined by the balance between numerous pro-angiogenic factors and anti-angiogenic factors. Increase of angiogenic activity has been regarded as the result of an alteration between the balance of pro- and anti-angiogenic factors,
which might be caused by increase of the pro-angiogenic factor, decrease of the anti-angiogenic factor, or both. Many OA studies have referred to the presence of pro-angiogenic factors in degenerated cartilage, but to clarify the pathogenesis of angiogenesis at osteochondral junction in OA, prior to investigating each factor individually, angiogenic activity of the tissue as a whole have to be studied. It would be more important to focus angiogenic activity at osteochondral junction of OA rather than to reveal the presence of one or several pro-angiogenic factors. On the basis of this concept, oncological studies have dealt with angiogenic activity since the growth, invasion and metastasis of the tumor are angiogenesis-dependent.

In addition, considering angiogenesis as a target of treatment for OA, it would be far more valuable to identify the elevated angiogenic activity period rather than the degree of vascularity. In this study, longitudinal angiogenic activity of subchondral bone and cartilage were examined using a rabbit anterior cruciate ligament transaction (ACL) model. Associations between histological changes, angiogenic activity, and vascular invasion were also evaluated. We hypothesized that there would be a specific period during OA progression in which angiogenic activity increased followed by an increase in osteochondral vascular invasion.

Material and methods

OA models

New Zealand white rabbits (12-month-old) female weighing approximately 4 kg (SLC, Inc. Japan) were housed singly in cages in sanitary ventilated animal rooms with controlled temperature and humidity and regular light cycles. All rabbits were anesthetized with intramuscular injection of ketamine (100 mg/kg) and xylazine (5 mg/ml). Both knees were shaved and disinfected with isodine. ACLT was performed as described by Yoshioka et al. Briefly, a medial parapatellar incision was made and an arthroscopy was performed. The patella was dislocated laterally and the knee placed in full flexion. The ACL was visualized and transected with a No.12 blade. An anterior drawing test was performed gently to confirm that the ACL was transected completely. The joint was irrigated with sterile saline and closed. A sham operation was performed in the contralateral knee. The knee was opened and the patella was dislocated. After performing the anterior drawing test gently, the joint was irrigated and closed. After the operation, free patella was dislocated. After performing the anterior drawing test performed in the contralateral knee. The knee was opened and the cartilage was irrigated with sterile saline and closed. A sham operation was performed in the contralateral knee.

Macroscopic scoring

Gross morphological evaluation was performed in the MFC and the LFC to assess the macroscopic changes in articular cartilage using an established grading system involving Indian ink. Briefly, morphology was classified into four grades: a grade 1 (intact surface) surface is normal in appearance and does not retain Indian ink, a grade 2 (minimal surface) surface retains Indian ink as elongated specks or light gray patches, grade 3 (overt fibrillation) areas are velvety in appearance and retain ink as intense black patches, and grade 4 (erosion) areas are characterized by cartilage loss that exposes the underlying bone.

Histology

Histological evaluation was performed using haematoxylin/eosin (H/E) and Safranin-O Fast Green (SO) for each animal. Both femora and tibiae were cleaned and fixed with 4% paraformaldehyde for 5 days. After fixation, joints were dehydrated and delipidated with ethanol, and decalcification was performed using 20% ethylenediaminetetraacetic acid (EDTA) for 7 days. Decalcification was confirmed by radiograph. To evaluate the medial and lateral tibiofemoral joints, joints were embedded as coronal sections in paraffin blocks. Sections were cut at 5-µm thickness through the midpoint of the joint. Sections were deparaffinized using xylene and ethanol, and stained by H/E or SO to evaluate OA changes.

Immunohistochemistry was performed to identify vascular endothelial cells (ECs). ECs were visualized with a monoclonal anti-CD31 (platelet-endothelial cell adhesion molecule-1: PECAM-1) antibody using avidin-biotinylated peroxidase complex (ABC)-alkaline phosphatase (AP) methodology. Briefly, paraffin-embedded tissue section slides were prepared according to a routine procedure and cut into 5-µm sections (heat antigen retrieval was performed). After inhibiting endogenous peroxidase with 3% hydrogen peroxide, sections were incubated with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature to block nonspecific binding. Subsequently, the sections were incubated with primary rat monoclonal anti-CD31 antibody (Abcam, USA) at 4°C overnight. Sections were incubated for 30 min at room temperature with biotin-conjugated goat anti-rat secondary antibody and then incubated with avidin/streptavidin-horseradish peroxidase (HRP) under the same conditions. To visualize antigen localization, metal-enhanced 3, 3′-diaminobenxidine tetrahydrochloride (DAB) substrate (ImmuNoPure, Metal Enhanced DAB Substrate Kit, Pierce, Rockford, IL) was used. Sections were washed, dehydrated, and mounted under coverslips.

Quantification

Histological changes of articular cartilage were assessed using the Osteoarthritis Research Society International (OARSI) histochmical/histological grading system. This grading system assigns scores based on SO staining, changes of the cartilage surface, chondrocyte density, and cluster formation. Scores range from 0 to 24, with 0 representing normal cartilage and higher scores indicating progressive OA changes. Briefly, SO staining was assessed by area and depth, for which staining is scored from 0 (normal) to 3 (more than 8). Chondrocytes were assessed by chondrocyte count, which ranges from 0 (normal) to 4 (diffuse decrease). Cluster formation depends on the number of clusters, with scores ranging from 0 (normal) to 3 (more than 8).

Vascular density of osteochondral junctions

Vascular density of osteochondral junction was determined by counting the number of vessels crossing the osteochondral junction; i.e., the number of vessels contacting or crossing the tidemark was counted along the entire MFC or the LFC. An average of five coronal sections of weight-bearing area, harvested at 100 µm interval, was calculated for each knee.

Angiogenesis assay

A co-cultured tubule formation assay was conducted to evaluate angiogenic activity of specimens. Human umbilical vein endothelial cells (HUVECs) and human diploid fibroblasts (HDFs)
were purchased (Angiogenesis Kit, KURABO, Japan) and co-cultured with specimens according to the manufacturer’s instructions. Briefly, HUVECs and HDFs were mixed and seeded in each individual culture well (16-mm diameter) of a 24-well plate, and the specimens placed in the cell insert with a 0.45-μm membrane (Intercell, KURABO, Japan) were added and co-cultured. This cell insert allowed permeation of the active substances produced by the specimens but did not allow direct contact with cells. Co-cultured cells were incubated in endothelial culture medium for 10 days at 37°C in 5% CO₂ in humidified air, and culture medium was exchanged every 2–3 days. On day 11, the insert was removed and vessel formation was evaluated.

Subchondral bone and cartilage were obtained from the MFC and the LFC, as well as the synovium. Cartilage was removed by scalpel and subchondral bone of the weight-bearing area was resected. Total cartilage from each condyle was gathered and the subchondral bone of the weight-bearing area was cut into 3 mm-thickness square of 10 mm on a side to make an equivalent sample size. Synovium weighing 20 mg was also collected. Then they were placed separately into cell inserts, which were placed in each well.

After 11 days of culture, tubes were immunostained according to the manufacturer’s instructions and analyzed with photomicrographs using computer software (Angiogenesis Image Analyzer, KURABO, Japan). Briefly, tubes were fixed with 70% ice-cold ethanol and immunostained with a mouse anti–PECAM-1 (CD31) antibody to visualize tube formation. Cells were washed with PBS containing 2% BSA and incubated with mouse anti–human PECAM-1 antibody for 1 h at 37°C. Wells were washed and incubated with AP-conjugated anti-mouse antibody (R&D Systems, Abingdon, UK) and stained using 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT).

**Image analysis**

The whole area of each well was captured by digital camera (Powershot, Canon, Japan) and saved as JPEG images. Photomicrographs were quantified using Angiogenesis Image Analyzer (KURABO, Japan). Images of tube cells were imported into the software and converted to binary format. The binary threshold was adjusted to obtain the best contrast of tubules with the background and was set for all of the images. The tube area was calculated.

Four variables (area, length, number of joints, and number of paths) related to tubule formation were calculated for each well. These four variables were compared between each time point and between each sample. To correct for individual differences, the ratio of OA/sham value was calculated.

**Statistical analysis**

Statistical comparisons were generated using Statview (SAS Institute Inc., NC). For detecting time-dependent changes in each parameter, Friedman test was performed to determine differences in each parameter: i.e., (1) OARSI scores of the MFC and the LFC from ACLT knees and those from sham knees; (2) numbers of vessels of the MFC and the LFC from ACLT knees and those from sham knees; (3) variables related to angiogenic assay of subchondral bone, cartilage, and synovium in each knee. For comparison among OARSI scores and number of vessels of the MFC and the LFC from both ACLT and sham knees in each time point, a Kruskal–Wallis test was used. When significant differences were observed, subsequent post hoc comparisons were performed using Steel–Dwass tests. The results are expressed in the text as median [interquartile range (IQR)], and, for clarity, graphically as mean (95% confidence interval). Differences were considered significant when P-values < 0.05.

**Results**

**Gross morphology**

All ACLT knees demonstrated osteoarthritic changes, and the severity of OA changes increased in a time-dependent manner. The macroscopic grade of articular cartilage increased in a time-dependent manner for both the MFC and the LFC, although changes were severer in the MFC [Fig. 1(A)].

Minimal fibrillation was observed in the MFC 2 weeks after ACLT, while no change was detected in the LFC. At 4 weeks [Fig. 1(B)], overt fibrillation was observed in the MFC, while minimal fibrillation was noted in the LFC. Osteophyte formation was observed in the intercondylar fossa. At 6 weeks, cartilage erosion was noted in the MFC but not in the LFC. At 8–12 weeks, full thickness ulceration was observed in the MFC and erosion was seen in the restricted area of the LFC. Osteophyte formation was observed in the femoral condyle.

**Histological scoring**

Features characteristic of OA, such as loss of surface integrity, decreased number of chondrocytes, cluster formation, and loss of...
cartilage SO stain, increased with time primarily in the MFC. In the LFC, those changes followed about 4-week late [Fig. 2(A)]. The OARSI score of the MFC significantly increased in a time-dependent manner (4 weeks: \( P = 0.002 \), 6 weeks: \( P = 0.01 \), 8 and 12 weeks: \( P = 0.003 \)) as illustrated in Fig. 2(B), i.e., 2 weeks (median = 3, IQR: 2–3), 4 weeks (median = 6, IQR: 6–7), 6 weeks (median = 8, IQR: 7–9), 8 weeks (median = 10, IQR: 9–11), 12 weeks (median = 16, IQR: 15.5–18.5). In contrast, LFC score increased significantly at 8 weeks (\( P = 0.002 \)), i.e., 2 weeks (median = 1, IQR: 1–2), 4 weeks (median = 1, IQR: 1–2), 6 weeks (median = 3, IQR: 1–3), 8 weeks (median = 6, IQR: 6–7), 12 weeks (median = 6, IQR: 6–8). The MFC score was significantly higher in ACLT knees than in sham knees at each time point (2 weeks: \( P = 0.01 \), 4 weeks: \( P = 0.001 \), 6–12 weeks: \( P = 0.002 \)). The LFC score was also significantly higher in ACLT knees than in sham knees from 8 weeks (\( P = 0.002 \)). Furthermore, in ACLT knees, MFC score was significantly higher than LFC score at each time point (2 weeks: \( P = 0.02 \), 4 weeks: \( P = 0.004 \), 6–12 weeks: \( P = 0.002 \)). Sham knees showed no significant changes at any time point.

**Osteochondral vascularity**

Vascular invasion at the osteochondral junction of the MFC and the LFC significantly increased in ACLT knees compared to sham knees after surgery (\( P < 0.001 \)), as shown in Fig. 3. In the MFC, the degree of vascular invasion increased in a time-dependent manner, and the increase was significant from 6 weeks (\( P = 0.03 \)) to 8 weeks (\( P = 0.003 \)) compared to the immediately previous time points, i.e.,

**Fig. 2.** Histochemical/histopathological analysis. (A) Histological changes of the femoral condyle of the ACLT specimens. Loss of surface integrity, loss of chondrocytes, and loss of SO staining of cartilage, increased with time primarily in the MFC (a–c), as well as in the LFC (d–f). MFC showed loss of SO staining and started to loss surface integrity (black arrowheads) at 4 weeks (a). Erosion (white arrowheads) was noted at 8 weeks (b) and progressed at 12 weeks (c) in the MFC. Minimum loss of SO staining was noted from 4 weeks in the LFC (d) and expanded in a time-dependent manner (e,f). At 12 weeks (f) in the LFC, loss of staining, fissures, and decreased chondrocytes were observed, but those areas were limited compared to that of the MFC. Scale bar denotes 200 \( \mu m \). (B) Time-dependent progression of OARSI histochemical/histological score (n = 3 each). The score significantly increased in a time-dependent manner in the MFC from 4 weeks (2–4 weeks: \( P = 0.002 \), 4–6 weeks: \( P = 0.01 \), 6–8 weeks: \( P = 0.003 \), 8–12 weeks: \( P = 0.003 \)) after ACLT, and from 8 weeks in the LFC (\( P = 0.002 \)). The MFC had a significantly higher score than the LFC at each time point after 2 weeks (2 weeks: \( P = 0.02 \), 4 weeks: \( P = 0.004 \), 6–12 weeks: \( P = 0.002 \)). A significant difference was observed between ACLT knees and sham knees in the MFC at every time point after 2 weeks (2 weeks: \( P = 0.01 \), 4 weeks: \( P = 0.001 \), 6–12 weeks: \( P = 0.002 \)) and in the LFC at 8 and 12 weeks (\( P = 0.002 \)). Values are expressed as the mean and 95% confidence intervals for each group. Statistical significance relative to the immediately previous time point is indicated as: *\( P < 0.05 \), **\( P < 0.01 \). Statistical significance relative to the LFC in ACLT knees is indicated as: \( ^{\dagger} P < 0.05 \), \( ^{\ddagger} P < 0.01 \). Statistical significance relative to sham knees is indicated as: \( ^{\ast} P < 0.05 \), \( ^{\ast\ast} P < 0.01 \).

**Fig. 3.** (A) Vascular invasion in the osteochondral junction at 2, 4, 6, 8, and 12 weeks after ACLT (n = 3 each). Vascular invasion in subchondral bone of the MFC of ACLT knees significantly increased in a time-dependent manner from 6 (\( P = 0.03 \)) to 8 weeks (\( P = 0.003 \)) after ACLT. In the LFC of ACLT knees, vascular invasion significantly increased at 8 weeks (\( P = 0.04 \)). The degree of vascular invasion in ACLT knees was significantly larger in the MFC compared to the LFC at every time point after 2 weeks (2 weeks: \( P = 0.04 \), 4, 6 and 8 weeks: \( P = 0.002 \), 12 weeks: \( P = 0.004 \)). Vascular invasion in sham knees showed no time-dependent changes. Values are expressed as the mean with 95% confidence intervals for each group. Statistical significance relative to the immediately previous time point is indicated as: *\( P < 0.05 \), **\( P < 0.01 \). Statistical significance relative to the LFC in ACLT knees is indicated as: \( ^{\dagger} P < 0.05 \), \( ^{\ddagger} P < 0.01 \). Statistical significance relative to sham knees is indicated as: \( ^{\ast} P < 0.05 \), \( ^{\ast\ast} P < 0.01 \). (B) Representative histological image of vascular invasion in osteochondral junction of the MFC in ACLT knee. Vascular invasion increased in a time-dependent manner in the MFC of ACLT knees. (a) 2 weeks, (b) 4 weeks, (c) 8 weeks after ACLT. Sham knees indicated no vascular invasion breaching to the tidemark (d). Vasculature was confirmed by CD31 immunostaining. Immunopositive cells regarded as vascular ECs (e). Open arrowheads indicate vascular invasion at the osteochondral junction, white open arrows indicate the tidemark. Scale bar denotes 200 \( \mu m \) (a–d) and 50 \( \mu m \) (e) as well.
0 weeks (median = 3, IQR: 2–4), 2 weeks (median = 5, IQR: 4–6), 4 weeks (median = 7, IQR: 6.5–8), 6 weeks (median = 10, IQR: 10–11), 8 weeks (median = 16, IQR: 15–18), 12 weeks (median = 17, IQR: 15.5–21). After 8 weeks, no significant increase was observed. In the LFC, the degree of vascular invasion tended to increase in a time-dependent manner, although a significant increase was only observed at 8 weeks (P = 0.04), i.e., 0 weeks (median = 2, IQR: 2–3), 2 weeks (median = 3, IQR: 2–4), 4 weeks (median = 4, IQR: 3.5–4.5), 6 weeks (median = 5, IQR: 4–6), 8 weeks (median = 8, IQR: 7–8), and 12 weeks (median = 10, IQR: 7.5–10.5). The degree of vascular invasion in ACLT knees was significantly larger in the MFC than the LFC at every time point after 2 weeks (2 weeks: P = 0.04, 4, 6 and 8 weeks: P = 0.002, 12 weeks: P = 0.004). In contrast, no time-dependent changes were detected in sham knees.

**Angiogenic activity**

Tubule formation was accelerated with the subchondral bone of the MFC after 4 weeks of ACLT [Fig. 4(D)] whereas less accelerated after 12 weeks [Fig. 4(E)].

All the four variables showed a similar tendency for subchondral bone of the MFC and the LFC (Fig. 5).

Three out of four variables for subchondral bone of the MFC revealed a significant increase at 4 and 6 weeks after ACLT; AREA (P = 0.04), JOINT (P = 0.04) and PATH (P = 0.04) and then decreased at 8 and 12 weeks. The variable LENGTH showed similar tendency but it did not reach to a statistical significance.

All the four variables for subchondral bone of the LFC showed similar changes, although the changes occurred later than those for the MFC: they increased at 6 weeks (AREA: P = 0.04, LENGTH: P = 0.04, JOINT: P = 0.008, PATH: P = 0.04) and decreased at 8 weeks.

In contrast, variables for cartilage both of the MFC and LFC revealed no changes at any time point (Fig. 6).

**Discussion**

Angiogenesis in the osteochondral junction is considered to be an important factor in the pathogenesis of OA. This study demonstrated a time-dependent change of angiogenic activity of the subchondral bone, cartilage, and synovium in a rabbit OA model. To our knowledge, this is the first report to investigate the time-dependent changes of actual angiogenic activity of knee OA and to correlate them with histologically observed vascular invasion.

Angiogenic activity of subchondral bone showed a monotonic change during OA progression. In the subchondral bone of the MFC, angiogenic activity achieved a peak at 4 weeks after ACLT, and then decreased to baseline at 12 weeks. The subchondral bone of the LFC displayed the same tendency, although the timing was later than that for the MFC: angiogenic activity reached a peak at 6 weeks. Such time-dependent changes in angiogenic activity suggest a strong correlation between cartilage status and angiogenic activity, in which angiogenic activity reached a peak with minimum loss of surface integrity of cartilage and decreased SO stainability, and started to decrease to normal levels in the progressive to late stages of OA when obvious cartilage degradation observed.

Angiogenesis in the osteochondral junction, detected as vascular invasion from subchondral bone to cartilage, started to increase at 6 weeks in the MFC after ACLT and it continued to increase until 8 weeks, and the degree of vascular invasion was maintained after 8 weeks. In the LFC, it started to increase at 8 weeks and maintained at 12 weeks. Surge of vascular invasion appeared to start a little later than the increase in angiogenic activity. Considering the increased vascular invasion despite the decreased angiogenic activity in the later stages of OA, invaded

**Fig. 4.** Newly formed vessels (tubule formation) in a well were assessed after 11 days of culture using image software (Angiogenesis Image Analyzer, KURABO, Tokyo). (A) As the first step, tubule formation after co-culture of HUVECs, HDFs, and specimen were stained with anti-CD31 antibodies. (B) Then, the images of tubules were converted to a binary format using software. (C) The binary threshold was adjusted to obtain the best contrast of tubules with background. Variables related to tubule formation were calculated automatically using this image. (D) Representative image of accelerated tubule formation observed by co-culturing subchondral bone of the MFC at 4 weeks after ACLT. (E) Inhibited tubule formation with subchondral bone of the MFC at 12 weeks after ACLT.
vasculature appeared to be maintained as the resultant vasculature accumulated. Therefore, the increased degree of vascular invasion observed in the osteochondral junction of late phases of OA may only reflect what occurred during the course of development of OA.

Vascular invasion has been reported for both human OA as well as animal OA models. In human studies, several reports have described an increase of vascular invasion at the osteochondral junction in late stages of knee OA, and associated this with OA pathogenesis. However, these conclusions were based on histological evaluations that only assessed vascular invasion and not actual angiogenic activity. Compared to the present results, these previous results are expected, since only accumulated vasculature was detected. However, the angiogenic activity and vascular invasion that occur during human OA development require further elucidation.

In contrast, studies in animal models have reported that vascular invasion from subchondral bone to cartilage occurred in the early stages of OA. These studies indicated that vascular invasion into the articular cartilage was one of the earliest observed changes and contributed to other OA features. However, the results of the present study, these previous results are expected, since only accumulated vasculature was detected. However, the angiogenic activity and vascular invasion that occur during human OA development require further elucidation.

Angiogenic activity of the cartilage in both the MFC and the LFC showed no significant changes throughout the entire experimental period. Although vascular invasion to cartilage is considered to contribute to cartilage degeneration in OA, little is known about angiogenic activity in cartilage. Smith et al. investigated the anti-angiogenic properties of articular cartilage of human OA and concluded that loss of resistance to vascular invasion distinguished OA cartilage from normal cartilage. It might be possible that not only elevated angiogenic activity of subchondral bone but also loss of resistance to vascular invasion is required in actual vascular invasion at osteochondral junction in OA.

The angiogenic activity of the synovium was also investigated. Activity increased from 6 weeks after ACLT, and high activity was maintained at 8 and 12 weeks. This time-dependent change differed from that of subchondral bone where monomodal change was observed both in the MFC and the LFC. And our result suggested that angiogenic activity of subchondral bone depended on status of overlaying cartilage. Our result appeared as consistent with Walsh et al. who reported that osteochondral and synovial angiogenesis appear to be independent processes that contribute to OA pathogenesis in different manners.

The angiogenic activity of tissues depends on the balance of pro- and anti-angiogenic factors. Pro-angiogenic factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), interleukin (IL)-1, IL-8 has been reported to be expressed by articular chondrocytes and osteoblasts of subchondral bone in OA. The most extensively studied factor, VEGF, is the primary pro-angiogenic factor involved in angiogenesis in many tissues, including cartilage. However, time-dependent changes of VEGF expression remain controversial. Tibesku et al. reported...
that expression of VEGF by chondrocytes increased with OA progression in a rat model. On the other hand, Pickarski et al. reported using a rat model that VEGF was up-regulated 2–6 weeks after ACLT and decreased to sham level at 10 weeks.

The present results show a decrease of angiogenic activity of subchondral bone in the late phases of OA. This conclusion appears to contradict the results of most previous studies that reported up-regulated VEGF expression in late OA phases. However, considering that VEGF not only promotes angiogenesis but also plays an important role in the maintenance of established vasculature, and that angiogenic activity is not determined by a single factor, the presence of VEGF expression in the late phases of OA does not necessarily indicate elevated angiogenic activity.

Regarding anti-angiogenic factors, Hayami et al. evaluated the expression of chondromodulin-I (ChM-I) in a rat OA model. They observed decreased expression of ChM-I in the superficial zone of articular cartilage in advanced OA, leading to the conclusion that loss of ChM-I might be responsible for promoting vascular invasion into the cartilage during progression of OA. Franses et al. showed that chondrocytes in the superficial layer of OA displayed increased expression of VEGF as well as the anti-angiogenic factors tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-3, secretory leuko-pro tease inhibitor (SLPI), and plasminogen activator inhibitor-1 (PAI-1). However, expression of these factors was not associated with osteochondral vascular density, indicating that these factors did not contribute to the prevention of vascular invasion. These investigators concluded that the failure of deep chondrocytes to express anti-angiogenic factors might permit vascular invasion. Many factors are involved in angiogenesis, and little is known about the interactions between these factors. Further study is required to elucidate the main substance and the interaction between the substance involved in angiogenic activity when considering angiogenesis inhibition as a treatment strategy for OA.

Recently, a few reports about angiogenesis-targeted therapies for OA have been published. Mapp et al. used a matrix metalloproteinase (MMP) inhibitor in a rat OA model and reported a reduction in angiogenesis in osteochondral junctions, as well as articular cartilage damage. Ashraf et al. used PPI2458, an anti-angiogenic fumagillin analog, in a rat OA model, and indicated that it reduced synovial and osteochondral angiogenesis, synovial inflammation, joint damage, and pain. These studies initiated administration of these compounds within a maximum 11 days after surgery, which most likely represented early stages of OA, independent of which surgical procedure was used. Since angiogenic activity is elevated in early stages of OA, and not in late stages as shown in the present study, the time window for administration of anti-angiogenic agents may be narrow. Combining the results of the current and previous studies, we suggest that anti-angiogenic agents may only be effective when administered during relatively early-stage OA.

The present study has a few limitations, which are common to other investigations in animal OA models. First, the resected and cultured samples did not simulate physiological intra-articular conditions. The specific conditions, under which angiogenic factors are generally expressed, such as hypoxia and load stimulation, were too complicated to simulate in this experimental model. Secondly, the time-series comparison was done at 2-week intervals. Smaller intervals might be required to accurately assess the detailed time-dependent changes in angiogenic activity. Lastly, only subchondral bone of the femur was assessed. We speculate that similar changes would be observed in the tibial side because deteriorations in the femur and tibia strongly related to one
another. But subchondral bone of the tibia might display different properties.

Conclusion

This study revealed time-dependent changes in angiogenic activity in subchondral bone in a rabbit ACLT model of OA. Angiogenic activity increased in the early and progressive phases and decreased in the late phases of OA. Increased vascular invasion followed the increase in angiogenic activity, indicating that angiogenic activity and the subsequent vascular invasion of subchondral bone to cartilage are early features of OA and contribute to the progression of OA.

Contribution

Masahiko Saito contributed to the design, acquisition of the data, analysis and interpretation, drafting and revising of the manuscript and final approval.

Takahisa Sasho contributed to the conception, design, critical revision, and final approval of the article and obtaining of funds for this study.

Kouichi Nakagawa, Kazuhiro Takahashi made contribution from the conception and design through drafting.

Nobuyasu Ochiai, Satoshi Yamaguchi, Junichi Nakamura and Arata Nakajima contributed to the critical revision and final approval of the manuscript.

Naoshi Ikegawa, Ryuichiro Akagi, Yuuta Muramatsu and Shunsuke Mukoyama contributed to the experimental surgery of the model, histological assessment and final approval of the manuscript.

Takahisa Sasho and Masahiko Saito take responsibility for the integrity of the work.

Role of the funding source

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Conflict of interest

All authors have no conflicts of interest.

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