View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Okayama University Scientific Achievement Repository

Acta Medica Okayama

Volume 51, *Issue* 5 1997 *Article* 1

OCTOBER 1997

Expression of osteonectin in articular cartilage of osteoarthritic knees

Yoshifumi Nanba[*] Keiichiro Nishida[†] Takeshi Yoshikawa[‡]

Toru Sato[**] Hajime Inoue[††] Yoshinori Kuboki[‡‡]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,
[**]Okayama University,
[††]Okayama University,
[‡‡]Hokkaido University,

Copyright ©1999 OKAYAMA UNIVERSITY MEDICAL SCHOOL. All rights reserved.

Expression of osteonectin in articular cartilage of osteoarthritic knees*

Yoshifumi Nanba, Keiichiro Nishida, Takeshi Yoshikawa, Toru Sato, Hajime Inoue, and Yoshinori Kuboki

Abstract

The expression of osteonectin (ON) in osteoarthritic articular cartilage was investigated by enzyme immunohistochemistry and colloidal gold immunoelectron microscopy. A total of 96 specimens from 9 knees of 8 patients with osteoarthritis (OA) were examined. In OA cartilage, ON-positive cells varied in distribution and were not seen in all the specimens obtained from the same patient. However, in over half of the specimens (56 of 96), especially in the specimens of Mankin's grades from 4 to 9, which corresponds to relatively early stages of OA, ON was expressed in the cartilage above the calcified layer. On the other hand, ON was detected only in the calcified layer below the tidemark in normal articular cartilage. In addition, colloidal gold immunoelectron microscopy revealed ON in chondrocytes and matrix vesicles (MVs). These findings suggest that ON acts through MVs in the early stages of OA as a significant pathogenetic factor involved in intracartilage calcification, which is known to have a close relationship to the progression of OA.

KEYWORDS: chondrocalcinosis, osteoarthritis, osteonection, Mankin's histologic-histochemical grading, calcification

---

*PMID: 9359920 [PubMed - indexed for MEDLINE]
Copyright (C) OKAYAMA UNIVERSITY MEDICAL SCHOOL

ACTA MED OKAYAMA 1997; 51(5): 239-243

Expression of Osteonectin in Articular Cartilage of Osteoarthritic Knees

Yoshifumi NANBA*, Keiichiro NISHIDA, Takeshi YOSHIKAWA, Toru SATO, Hajime INOUE and Yoshinori KUBOKI[a]

Department of Orthopaedic Surgery, Okayama University Medical School, Okayama 700 and [a]Department of Biochemistry, School of Dentistry, Hokkaido University, Hokkaido 060, Japan

The expression of osteonectin (ON) in osteoarthritic articular cartilage was investigated by enzyme immunohistochemistry and colloidal gold immunoelectron microscopy. A total of 96 specimens from 9 knees of 8 patients with osteoarthritis (OA) were examined. In OA cartilage, ON-positive cells varied in distribution and were not seen in all the specimens obtained from the same patient. However, in over half of the specimens (56 of 96), especially in the specimens of Mankin's grades from 4 to 9, which corresponds to relatively early stages of OA, ON was expressed in the cartilage above the calcified layer. On the other hand, ON was detected only in the calcified layer below the tidemark in normal articular cartilage. In addition, colloidal gold immunoelectron microscopy revealed ON in chondrocytes and matrix vesicles (MVs). These findings suggest that ON acts through MVs in the early stages of OA as a significant pathogenetic factor involved in intracartilage calcification, which is known to have a close relationship to the progression of OA.

Key words: chondrocalcinosis, osteoarthritis, osteonectin, Mankin's histologic-histochemical grading, calcification

Although a matrix vesicle (MV) theory was published as early as 1960 as a means of explaining the calcification mechanism, a consistent theory explaining all the mechanisms of calcification has not yet been put forward. MVs are extra-cellular membrane organelles found primarily in the matrix of the epiphyseal cartilage (1, 2). MVs always appear in the region where calcification first starts in the mesenchymal tissue. They induce calcification through a series of mechanisms which transport calcium accumulated in the mitochondria and release it in the initial calcifying region (3-5).

In addition to MVs, several matrix proteins such as type X collagen and osteonectin (ON) seem to be associated with the process of calcification (6, 7). ON is a 32,000 dalton protein that binds selectively to both hydroxyapatite and collagen (7) and is known to occur about 20 % of non-collagenous osteoproteins (8). Recent immunohistologic studies have shown that ON is present in high concentrations in the newly mineralized matrix of fetal bones and also in the matrix of mineralizing hypertrophic cartilage (9). Its preferential localization to calcifying tissues and its high binding affinity to calcium and collagen type-1 strongly suggests that ON plays a unique role in the process of calcification (10).

In the course of osteoarthritis (OA), calcification in the articular cartilage (chondrocalcinosis) is frequently found. Although chondrocalcinosis seems to be a part of matrix repair, the mechanism still remains unclear. ON is a biosynthetic product of the articular cartilage (11) and may play a role in calcification of OA cartilage, but there has been no report concerning its distribution in OA cartilage. In the present study, the expression of ON in OA articular cartilage was studied immunohistochemically.

Materials and Methods

Tissues. Articular cartilage was obtained from 8 patients with OA during total-knee arthroplasty: one male knee and 8 knees from 7 females. The age at surgery of the patients varied from 58 to 80 years, with an average of 69. All the knees were radiologically classified to the last stage of OA with chondrocalcinosis using Ishikawa's criteria (12). A total of 96 specimens were prepared from the 9 knees.

Joint cartilage from 3 patients without joint disease

* To whom correspondence should be addressed.

Acta Medica Okayama, Vol. 51 [1997], Iss. 5, Art. 1

was used as a normal control: talus cartilage obtained from an above-knee amputation of a 16-year-old boy with osteosarcoma of the femur, femoral head cartilage obtained during prosthetic replacement of a 29-year-old man with neck fracture of the femur, and talus cartilage obtained from a below-knee amputation of a 42-year-old woman with polyarteritis nodosa.

Light microscopy. The tissue samples were fixed in 0.1 M phosphate-buffered 4 % paraformaldehyde solution for 2 h at room temperature, washed with 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 2 h, and decalcified by the Planc-Rychlo method using a solution containing 7.0 g $AlCl_3$, 8.5 ml HCl, 5.0 ml formic acid, and distilled water in 100 ml for 12 h at room temperature. Then, they were neutralized with a 5 % sodium sulfate solution for 1 h and embedded in paraffin. Paired sections were stained for light microscopy and also examined immunohistochemically.

Immunohistochemical staining. The avidin-biotin-peroxidase complex (ABC) method, using a rabbit anti-bovine ON antibody, was employed (13). This antibody cross-reacts with human ON (14). After deparaffination, sections were immersed in 3 % H_2O_2 in methanol to prevent endogenous peroxidase reactions and washed with distilled water 3 times. Then, they were blocked with 10 % normal goat serum (Vector Laboratories Inc, Burlingame, USA) for 60 min to eliminate non-specific reactions. An optimal concentration of rabbit antibody against bovine ON was then allowed to react with the specimens at 4 °C for 12 h. As a secondary antibody, biotinized goat anti-rabbit IgG serum (Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA) was added for 30 min at room temperature. After washing with PBS, ABC (Nichirei Corporation, Tokyo, Japan) was allowed to react for 30 min at room temperature. After washing, the specimens were immersed in 3,3'-diaminobenzidine H_2O_2 (0.01 %) for 5 min to develop reaction products and counterstained with hematoxylin. Control specimens were processed in the same fashion, excluding the primary antibody.

Safranin-O staining for light microscopy. Ordinary safranin-O staining was performed for the collected samples of articular cartilage. The severity of the OA condition was graded according to Mankin's histologic-histochemical grade which assigns scores to structure (0–6), cell distribution and density (0–3), safranin-O staining (0–4), and the integrity of the tidemark (0–1) (15). The total scores for the OA specimens examined in this study ranged from 2 to 12.

Colloidal gold immunoelectron microscopy. The cartilage specimens were fixed in 0.1 M phosphate-buffered 4 % paraformaldehyde and 0.1 % glutaraldehyde mixture for 4 h, washed with 0.1 M PBS containing 8 % sucrose, embedded in a hydrophilic resin (LR-White), and sectioned.

The anti-bovine ON antibody was allowed to react with the sections for more than 6 h. After washing 3 times, the tissue sections were reacted with goat anti-bovine IgG labeled with 5 nm colloidal gold particles (E.Y. Laboratories, Inc., San Mateo, USA) as the secondary antibody. They were stained with uranyl acetate-citric acid complex and examined with a Hitachi H7100 electron microscope.

Statistical analysis. The relationship between ON-positivity rates and Mankin's grade was statistically evaluated using the Mantel-Haenzsel method.

Results

Normal cartilage tissue. A total of 10 specimens were prepared from the articular cartilage of the control patients. They were evaluated to be Mankin's grade 0–1 and defined as histologically normal. In the control group, only the matrix in the calcified layer below the tidemark showed a positive immunoreaction (Fig. 1).

Osteoarthritic cartilage tissue. Microscopically, chondrocytes expressing ON or showing a positive reaction in the pericellular matrix were evaluated as being ON-positive cells. ON-positive chondrocytes in the OA articular cartilage varied in distribution and were not always seen in different specimens obtained from the same individual. In over half of the specimens (56 of 96), however, ON appeared in the deeper layer above the tidemark (Fig. 2a, b). High magnification showed that positive reactions were also seen in the area extending from the territorial resion to the interterritorial resion surrounding the positive chondrocytes (Fig. 2c). Because ON was seen only in the calcified layer below the tidemark in the normal cartilage, immunoreactivity above the tidemark was defined as using a positive immunoreaction to ON.

Evaluation of the relationship between ON-positivity rates and Mankin's histologic-histochemical grading of OA cartilage revealed that a positive immunoreaction to ON was observed more frequently in the specimens of grades from 4 to 9, which correspond to the stages of

http://escholarship.lib.okayama-u.ac.jp/amo/vol51/iss5/1

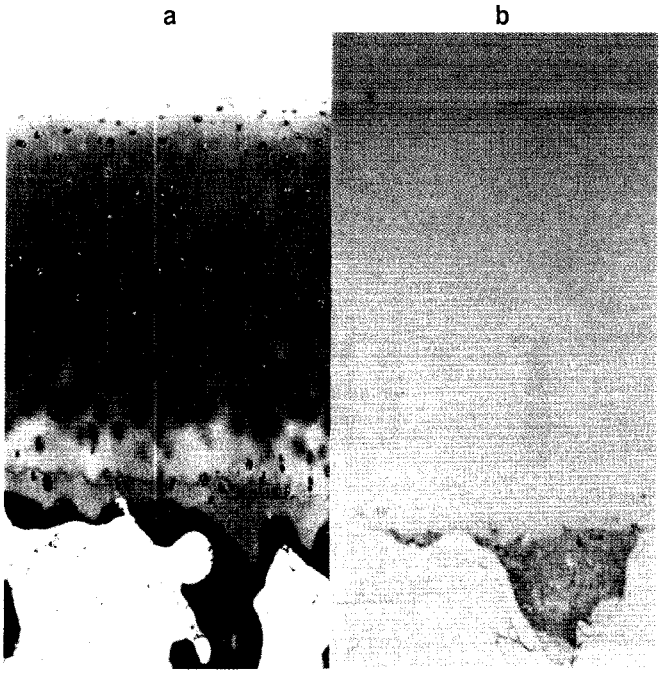

Fig. 1 Articular cartilage of a 16-year-old patient with osteosarcoma.
a) Safranin-O staining. The staining pattern was evaluated to be grade 1 according to Mankin's histologic-histochemical grading. × 40.
b) Immunohistochemical staining. Expression of osteonectin (ON) was observed only in the calcified layer below the tidemark. × 40.

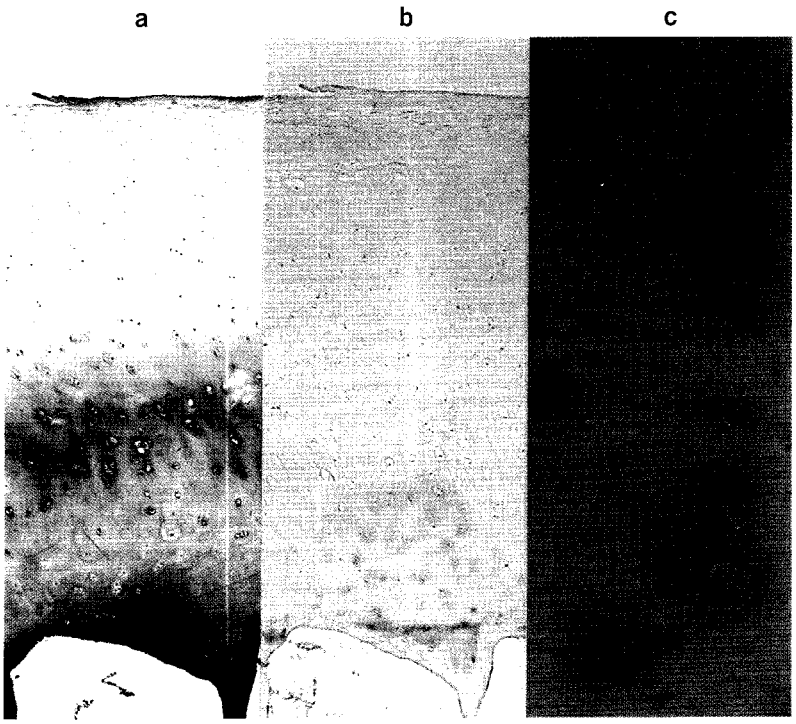

Fig. 2 Articular cartilage of a 65-year-old patient with osteoarthritis (OA) evaluated to be grade 6 according to Mankin's histologic-histochemical grading. **a**) Safranin-O staining. × 40. **b**) Immunohistochemical staining. ON-positive reaction was observed in the deep layer in addition to the calcified layer. × 40. **c**) Immunohistochemical staining. High magnification shows that positive reactions are seen in the area extending from the territorial region to the interterritorial region surrounding the positive chondrocytes. × 200. ON: See Fig. 1.

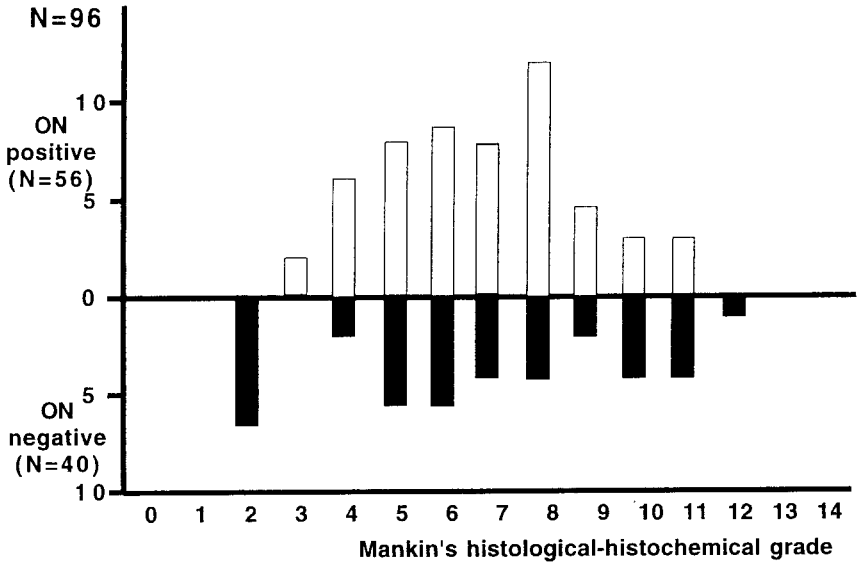

Fig. 3 The relationship between ON-positivity rates and Mankin's grading of OA cartilage. OA, ON: See legends to Figs. 1, 2.

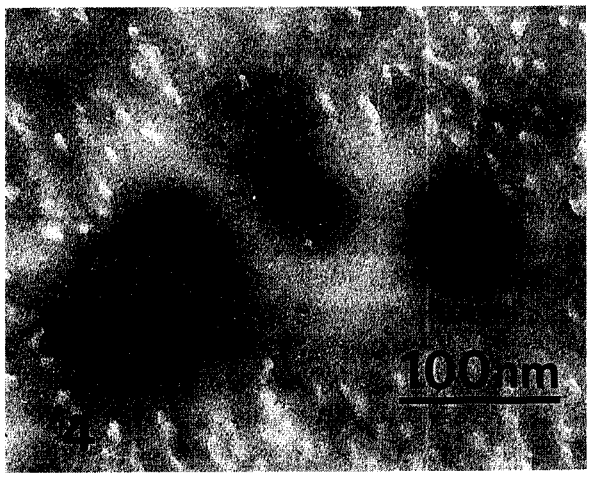

Fig. 4 Colloidal gold immunoelectron microscopy. Colloidal gold particles are deposited selectively in vesicular structures in the extracellular matrix which are considered to be matrix vesicles. This section was obtained from the same patient as in Fig. 2. × 40,000.

mild or moderate degeneration of the cartilage, than in those of the other grades (ON-positivity rate: 66.7 % *vs.* 33.3 %, $P = 0.0041$) (Fig. 3).

In transmission immunoelectron microscopy, ON was detected in the chondrocytes and the surrounding matrix. In the matrix, the deposition of colloidal gold particles was detected specifically in the vesicular structures which were considered to be MVs (Fig. 4).

Discussion

We investigated ON expression in OA cartilage and found that ON was present in the deeper layer above the tidemark. This is in contrast to ON expression in normal cartilage, in which ON appears only in the calcified layer below the tidemark, a pattern we confirmed in our study (14). Ours is the first report to clarify the expression patterns of ON in OA cartilage, to our knowledge.

OA often accompanies chondrocalcinosis which is one of the pathological findings of articular cartilage degeneration. This change begins with calcification of the meniscus and is thought to cause the formation of pseudo-gout when calcium pyrophosphate (CPPD) crystals are released into the articular cavity, resulting in inflammation of the joint. Light and electron microscopy of the tissues collected from OA joints revealed CPPD crystals in 45 % of them, and a close relationship between chondrocalcinosis and OA has been suggested (16).

Once breakdown of the matrix occurs, the metabolism of chondrocytes becomes abnormal. It has been shown

that chondrocytes produce various cytokines and enzymes such as matrix metalloproteinases (MMPs) (17) which are believed to play a central role in the cartilage breakdown. According to a report by Okada, more MMP-3 positive cells appear in the early stages of OA (up to Mankin's grade 7) than in later stages (grades 8 to 14) (18). The same tendency of ON expression was observed in our study: we observed higher ON-positivity rates in the early stages of cartilage degeneration (Mankin's grades 4 to 9) OA chondrocytes may produce ON and other chemical substances such as MMPs in excess under early accelerated metabolism and ON, which traps calcium in MVs, may regulate calcification. Then, chondrocalcinosis proceeds to such an extent that calcium deposits can be observed roentogenographically. On the other hand, other mediators, which influence the expression of ON, are synthesized in the course of OA degeneration; probably ON could neither exist nor function properly in the severely affected OA cartilage.

Corticosteroids have a strong anti-inflammatory action and are often injected intra-articularly, but they are also known to induce chondrocalcinosis (12). Generally, steroids inhibit the secretion of IL-1 by macrophages and monocytes. Recently, Chandrasekhar *et al.*, reported that the production of ON is inhibited specifically by IL-1 and is promoted by several growth factors (11). Based on our findings, it is possible that a mechanism mediated by IL-1, ON and several growth factors may affect chondrocalcinosis.

Acknowledgements. In closing, we would like to express our deep gratitude to Mrs. Fumiko Isobe, a technical assistant of the Department of Orthopaedics, Okayama University, for her cooperation in this study.

References

1. Anderson HC: Electron microscopic studies of induced cartilage development and calcification. J Cell Biol (1967) **35**, 81-101.
2. Bonucci E: Fine structure of early cartilage calcification. J Ultrastr Res (1967) **20**, 33-50.
3. Wuthier RE: Proposed mechanism of matrix vesicle formation and vesicle-mediated mineralization; in Matrix Vesicles, Ascenzi A, Bonucci E and Bernard B eds, Wichtig Editore srl, Milano (1981) p103.
4. Anderson HC: Mineralization by matrix vesicles. Scanning Electron Microsc (1984) **2**, 953-964.
5. Sumii H and Inoue H: Ultrastructure and microanalysis of epiphyseal growth cartilage of femoral head processed by rapid-freezing and freeze-substitution. Acta Med Okayama (1993) **47**, 95-102.
6. Gordon DW, Fischer M, Gannon J, Thompson RC Jr and Oegema TR Jr: Expression of type-X collagen in osteoarthritis. J Orthop Res (1995) **13**, 4-12.
7. Pacifici M, Oshima O, Fischer LW, Young MF, Shapiro IM and Leboy PS: Changes in osteonectin distribution and associated with mineralization of the chicken tibial growth cartilage. Calcif Tissue Int (1990) **47**, 51-61.
8. Lane TF and Sage EH: The biology of SPARC, a protein that modulates cell-matrix interactions. FASEB (Fed Am Soc Exp Biol) J (1994) **8**, 163-173.
9. Jundt G, Berghauser KH, Termine JD and Schulz A: Osteonectin: A differentiation marker of bone cells. Cell Tissue Res (1987) 248, 409-415.
10. Romberg RW, Werness PG, Lollar P, Riggs BL and Mann KG: Isolation and characterization of native adult osteonectin. J Biol Chem (1985) **260**, 2728-2736.
11. Chandrasekhar S, Harvey AK, Johnson MG and Becker GW: Osteonectin/SPARC is a product of articular chondrocytes/cartilage and is regulated by cytokines and growth factors. Biochim Biophys Acta (1991) 122, 7-14.
12. Ishikawa K: A study of deleterious effects of intraarticular corticosteroid on knee joints: A clinical investigation on primary gonoarthrosis. J Jpn Orthop Assoc (1978) **52**, 359-374 (in Japanese).
13. Kuboki Y, Takita H, Komori T, Mizuo M, Furu-uchi E and Taniguchi K: Separation of bone matrix protein by calcium-induced precipitation. Calcif Tissue Int (1989) **44**, 269-277.
14. Chiba H and Matsuyama T: Immunohistochemical localization of bone gla protein and osteonectin in normal human bone and cartilage tissues, and in chondro- sarcomas. J Jpn Orthop Assoc (1993) **67**, 463-472 (in Japanese).
15. Mankin HJ, Dorfman H, Lippiello L and Zarins A: Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. J Bone Jt Surg Am Vol (1971) **53**, 523-536.
16. Ishikawa K: Osteoarthritis of the knee and articular chondrocalcinosis. J Jt Surg (1985) **4**, 725-739 (in Japanese).
17. Shinmei M, Masuda K, Kikuchi T and Shimomura Y: Interleukin-1, tumor necrosis factor and interleukin-6 as mediators of cartilage destruction. Semin Arthritis Rheum (1989) **18**, 27-32.
18. Okada Y: Matrix metalloproteinases and the inhibitor of osteoarthritis. Off J Jpn Rheum Assoc (1994) **33** (Suppl) 488 (in Japanese).

Received January 20, 1997; accepted March 31, 1997.