Osteoarthritis and Cartilage (2002) **10,** 180–187
© 2002 OsteoArthritis Research Society International
doi:10.1053/joca.2001.0505, available online at http://www.idealibrary.com on IDEAL®
1063–4584/02/030180+08 $35.00/0

Osteoarthritis and Cartilage
Journal of the OsteoArthritis Research Society International

Development and regulation of osteophyte formation during experimental osteoarthritis

S. Hashimoto*, L. Creighton-Achermann*, K. Takahashi†, D. Amiel†, R. D. Coutts†‡ and M. Lotz*
**Division of Arthritis Research, The Scripps Research Institute, La Jolla, California, U.S.A.*
†Department of Orthopedics, University of California, San Diego, California, U.S.A.
‡The Malcolm and Dorothy Coutts Institute for Joint Reconstruction and Research, San Diego, California, U.S.A.

Summary

Objectives: Osteophytes represent areas of new cartilage and bone formation in human and experimentally induced osteoarthritis (OA). The present study addressed the production of nitric oxide (NO), vascular endothelial growth factor (VEGF) and the occurrence of apoptosis during osteophyte formation.

Design: Osteophytes in the knee joint of rabbits that developed OA-like lesions following anterior cruciate ligament transection (ACLT) were analysed by histology and immunohistochemistry for NO production, and the presence of VEGF. TUNEL was used to detect DNA fragmentation.

Results: At the joint margins in the interface between cortical bone marrow and periosteal lining growth plate-like formations were detectable as early as 4 weeks after ACLT. By 12 weeks after ACLT osteophytes were visible in 100% of femoral condyles and tibial plateaus. Discrete areas with proliferating chondrocytes, hypertrophic chondrocytes, calcified matrix and vascular invasion were observed. VEGF immunoreactivity was most prominent in hypertrophic chondrocytes 9 weeks after ACLT. Nitrotyrosine immunoreactivity was detected in endothelial cells and in some hypertrophic chondrocytes in the calcified zone 4 weeks after ACLT. After 8 and 12 weeks, positive cells were detected in the hypertrophic and calcified zone. TUNEL-positive cells were seen in blood vessels, and among hypertrophic chondrocytes adjacent to the blood vessels 4 weeks after ACLT. The proliferative zone, pre-hypertrophic zone and hypertrophic zone showed only a few TUNEL positive cells. In contrast, 8 weeks and 12 weeks after ACLT, most hypertrophic chondrocytes, but few proliferative chondrocytes showed DNA fragmentation.

Conclusions: Hypertrophic chondrocytes in osteophytes express VEGF and this can promote vascular invasion of cartilage. The presence of TUNEL-positive cells shows a similar distribution as nitrotyrosine immunoreactivity during all phases of osteophyte development, suggesting that NO production and chondrocyte death are related events in osteophyte formation. © 2002 OsteoArthritis Research Society International

Key words: Osteophyte, Osteoarthritis, Nitric oxide, Apoptosis.

Introduction

Joints affected by osteoarthritis (OA) are not only characterized by the degradation of existing cartilage matrix but also by the production of new connective tissue in the form of osteophytes on the joint surface and more notably at the joint margins[1]. Osteophytes, also referred to as chondro-osteophytes, are also observed in experimentally induced arthritis[2–4]. Osteophytes can limit joint movement and represent a source of joint pain[5]. Their formation has been interpreted as an adaptation of the joint to the altered biomechanics of OA joints[6,7]. However, it is important to note that when osteophytes appear in the absence of other bone changes, they may not be a manifestation of OA and not predict radiographic progression of joint damage[8]. Osteophytes have also been detected in the intercondylar notch of human knees that did not show OA cartilage degradation[9] and in patients with anterior cruciate ligament rupture, osteophytes developed before changes in joint space width were detected[10].

The initial stimuli for osteophyte formation are not defined but mechanical[11,12] and humoral[13] factors are probably involved. Repeated injections of mouse joints with TGFβ or BMP[14] induced osteophytes or enhanced osteophyte formation in animals with experimentally induced OA. After repeated injections into normal mouse joints, both BMP-2 and TGFβ1 induced chondrogenesis at specific sites. Chondrophytes induced by BMP-2 were found predominantly in the region where the growth plates meet the joint space, while those triggered by TGFβ1 originated from the periosteum also at sites remote from the growth plates[14,15]. TGFβ, bFGF and IGF-1 were detected in osteophytes[16–18].

Osteophytes are composed of cells that express type I procollagen mRNA, mesenchymal prechondrocytes that express type IIA procollagen mRNA, and maturing chondrocytes that express type IIB procollagen mRNA. Based on the spatial pattern of gene expression and cytomorphology, the neochondrogenesis associated with osteophyte formation closely resembles that of healing

Received 13 April 2001; revision requested 16 September 2001; accepted 24 October 2001.

Address correspondence to: M. Lotz, Division of Arthritis Research – MEM 161, The Scripps Research Institute, La Jolla, CA 92037, U.S.A. Tel: 858-784 8960; Fax: 858-784 2744; E-mail: mlotz@scripps.edu

Table I
Gross morphology OA grade and osteophyte

	0 weeks	4 weeks	8 weeks	12 weeks
Femoral condyle				
OA grade	I: 100%	II: 60%, III: 40%	III: 50%, IV: 50%	II: 10%, III: 30% IV: 60%
Osteophyte	L: 0%, M: 0%	L: 100%, M: 100%	L: 100%, M: 100%	L: 100%, M: 100%
Tibial plateau				
OA grade	I: 100%	I: 60%, II: 20% III: 20%	II: 30%, III: 60% IV: 10%	III: 40%, IV: 60%
Osteophyte	L: 0%, M: 0%	L: 80%, M: 60%	L: 90%, M: 90%	L: 100%, M: 100%

The frequency of osteophytes and the severity of cartilage degradation was assessed 0, 4, 8 and 12 weeks after ACLT. L: lateral; M: medial.
The results are from the analysis of 6 rabbits per time point.

fracture callus[19–21] and is also similar to the epiphyseal growth plate[22]. An area of proliferating cells can be distinguished from layers with differentiating and terminally differentiated hypertrophic chondrocytes that express type X collagen. This area is vascularized and can undergo endochondral ossification[22,23].

Recent studies on growth plate have provided new insight into the cellular events and their regulation at the interface of hypertrophic cells and vascular invasion. Growth factors that regulate vascular invasion and chondrocyte hypertrophy have been defined. Vascular endothelial cell growth factor (VEGF) has been shown to regulate hypertrophic cartilage remodeling, ossification and vascular invasion of growth plate cartilage[24]. Vascular invasion is histologically linked to the development of apoptosis in differentiated chondrocytes of growth plate[25–27] and fracture callus[28,29]. Apoptosis is thought to represent a physiological event in the process of matrix mineralization, possibly through the formation of matrix vesicles[30].

Chondrocyte apoptosis also occurs in osteoarthritic articular cartilage and in this context is associated with matrix degradation and calcification[30–33]. Apoptosis can be induced in articular cartilage by nitric oxide[34]. OA cartilage contains iNOS expressing cells and produces NO spontaneously *in vitro*[35]. Nitrotyrosine immunoreactivity can be detected as an indicator of peroxynitrite formation[36]. The production of NO in growth plate and its relationship to apoptosis have not been examined.

The present study examined chondrocyte death, NO production and VEGF expression in osteophytes during experimentally induced OA in rabbits after anterior cruciate ligament transaction (ACLT).

Materials and methods

EXPERIMENTAL OSTEOARTHRITIS

Mature New Zealand White rabbits, aged 12 months were used in the present study. All rabbits were anesthetized by an intramuscular injection of ketamine (100 mg/kg) and zylazine (8 mg/kg). Knees were shaved and disinfected with Betadine solution. A medial parapatellar incision was made on the skin and a medial arthrotomy was performed. The patella was dislocated laterally and the knee placed in full flexion. The anterior cruciate ligament (ACL) was visualized and transected with a #15 blade. The joint was irrigated with sterile saline and closed. The capsule was closed with a running suture of 4-0 prolene and the skin was closed with a running mattress suture supplemented with interrupted sutures of 3-0 nylon. The contralateral side was not subjected to any surgical manipulation. Post-operatively, the animals were permitted cage (60 cm×60 cm×40 cm) activity without immobilization. The animals were closely monitored for infections and other complications. Cartilage was also harvested from normal rabbits that were not subjected to ACL transection.

GROSS MORPHOLOGY

Rabbits were sacrificed 4, 9 and 12 weeks after ACL transection. After sacrifice, both knees were harvested. The occurrence, site and severity of cartilage lesions and formation of osteophytes on the femoral condyles were mapped on a schematic representation of the rabbit knee. Gross morphological changes of the femoral condyles were assessed by established criteria[37] following the application of India ink: grade 1 (intact surface): surface appears normal and does not retain any ink; grade 2 (minimal fibrillation): site appears normal before staining, but retains the India ink as elongated specks or light gray patches; grade 3 (overt fibrillation): the cartilage is velvety in appearance and retains ink as intense black patches; grade 4 (erosion): loss of cartilage exposing the underlying bone.

TISSUE PROCESSING AND HISTOLOGY

Tissue blocks were fixed in 10% neutral buffered formalin with cetylpyridinium chloride (CPC) for 7 days and decalcified with EDTA. After decalcification (confirmed by X-ray), the femoral condyles were cut along the saggital plane and medial condyles were embedded in paraffin. Five micron sections were cut with a Reichert-Jung microtome and stained with hematoxylin and eosin (H&E), Safranin O/fast green under the following condition: dye concentration 0.1%, staining time 6 minutes, pH 5.7. All staining processes were performed at room temperature.

IN SITU DETECTION OF NDA FRAGMENTATION

Cartilage sections were fixed in 10% neutral buffered formalin for 15 h and embedded in paraffin. Sections (5 μm thickness) were floated onto poly-L-lysine coated slides. *In situ* detection of apoptosis was performed using the Apo

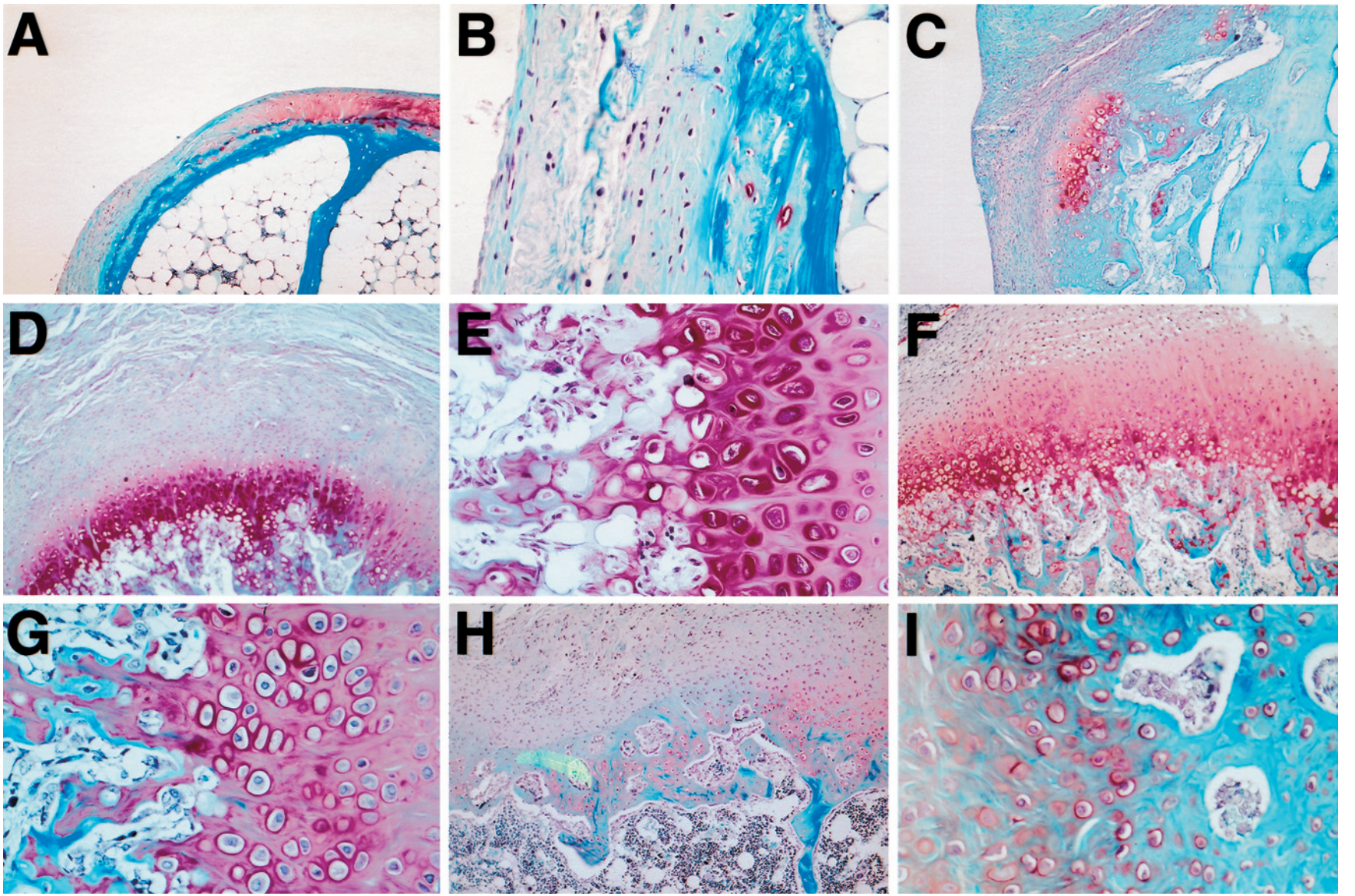

Fig. 1. Osteophyte formation in rabbit knees following ACL transection. Panel (a), (b): normal joints: Safranin O stain of transitional zone at joint margin from articular cartilage (red staining) to the fibrous safranin-O negative tissue. Note tidemark merging with bone at the lateral aspect of the joint [Panel (a) ×100; Panel (b) ×400]. Panels (c)–(e): 4 weeks after ACLT, proliferating chondrocytes, hypertrophic chondrocytes and matrix vascular invasion were observed at the joint margins [Panel (c) ×100; Panel (d) ×100; Panel (e) ×400]. Note absence of blue bone mineral staining in the cartilage adjacent to the blood vessels. Panels (f),(g): 9 weeks after ACLT the calcified zone appears enlarged and new bone tissue is observed [Panel (f) ×100; Panel (g) ×400]. Note blue bone mineral staining in the cartilage adjacent to the blood vessels. Panels (h),(i): 12 weeks after ACLT the calcified zone and new bone tissue are expanded, osteocytes, osteoblasts and osteoclasts can be identified in the new bone tissue [Panel (h) ×100; Panel (i) ×400].

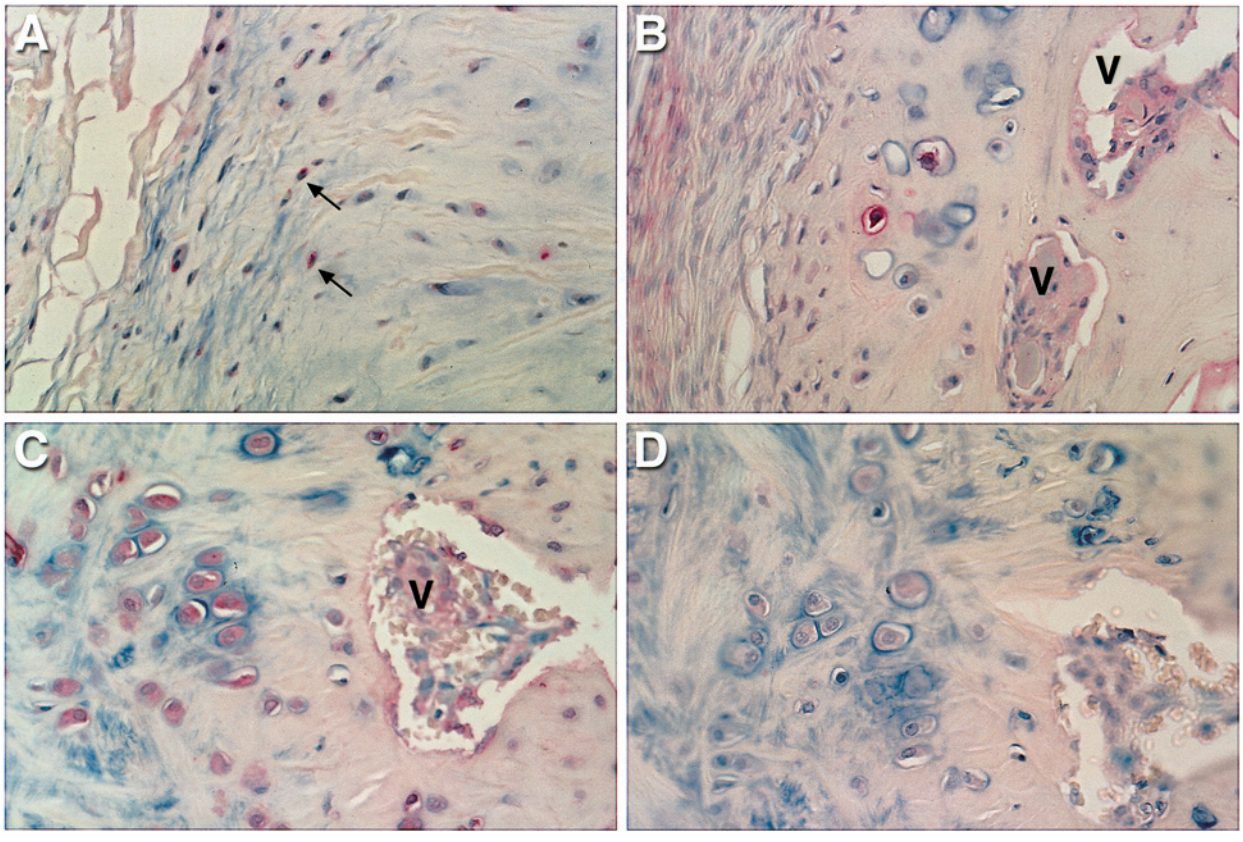

Fig. 2. VEGF expression in osteophytes. Panel (a): Normal joints: a small number of cells in the synvovial lining express VEGF (×400). Panel (b): 4 weeks after ACLT: hypertrophic chondrocytes in the hypertrophic zone of osteophytes adjacent to new blood vessels express VEGF (×400; V: blood vessel). Panel (c): 9 weeks after ACLT large numbers of hypertrophic chondrocytes in osteophytes express VEGF (×400). Panel (d): Osteophyte 4 weeks after ACLT, control stain with rabbit pre-immune IgG (×400).

Tag kit (Oncor, Gaithersburg, MD). In brief, sections were digested with proteinase K (20 μg/ml) for 15 min at room temperature. Sections were then pre-treated with bovine testicular hyaluronidase (0.5 mg/ml at 37°C for 30 min). DNA was end-labeled with digoxygenin-labeled dUTP using terminal transferase and detected with peroxidase-conjugated anti-digoxigenin antibody (Oncor). In the negative control terminal transferase was omitted. Adjacent sections were stained with H&E and Safranin O.

IMMUNOHISTOCHEMISTRY FOR NITROTYROSINE AND VEGF

Sections were pretreated with bovine testicular hyaluronidase (0.5 mg/ml at 37°C for 30 min) and incubated in 5% normal goat serum at room temperature for 20 min. Presence and distribution of nitrotyrosine were determined by the avidin/biotin peroxidase (ABC) method using rabbit anti-nitrotyrosine antibody (Upstate, Lake Placid, NY) and anti-VEGF (Oncogene, Cambridge MA) as primary antibody. The antibody was generated by immunizing mice with human recombinant $VEGF_{121}$. It recognizes proteins of 34–50 kDa identified as the various isoforms of VEGF. A biotinylated goat antirabbit antibody (Vector Laboratories, Burlingame, CA) was used as secondary antibody. Levamisole was added to block endogenous alkaline phosphatase. The negative control consisted of non-immune rabbit serum as a substitute for the primary antibody.

Results

THE DEVELOPMENT OF OSTEOPHYTES FOLLOWING ANTERIOR CRUCIATE LIGAMENT TRANSECTION

Four weeks after ACLT, osteophytes were visible at 100% of femoral condyles, 80% of the lateral tibial plateaus and in 60% of the medial tibial plateaus by gross morphological observation (Table I). In normal rabbit knees a transition from cartilage to perichondrium and periosteum is seen at the joint margin [Fig. 1(a),(b)]. Already, 4 weeks after ACLT, new cell formations were observed at the interface between bone marrow and the inner aspect of the periosteum [Fig. 1(c),(d)]. Cells were aligned in columns and cell size increased towards the bone marrow. New blood vessels intersected the area of hypertrophic cells [Fig. 1(e)]. This overall structure was similar to the epiphyseal growth plate. Nine weeks after ACLT osteophytes were seen in 90% of tibial plateaus and their size was increased (Table I). Newly formed bone tissue was observed at the calcified zone [Fig. 1(f),(g)]. After 12 weeks, osteophytes were visible in all femoral condyles and tibial plateaus (Table I). The calcified zone and new bone tissue were further enlarged and osteoblasts and multinucleated osteoclast-like cells could be distinguished at the leading front of vascularization [Fig. 1(h),(i)].

VEGF EXPRESSION IN CHONDROCYTES AND ANGIOGENESIS

VEGF has recently been shown to be expressed in hypertrophic cartilage in the growth plate and to coordinate

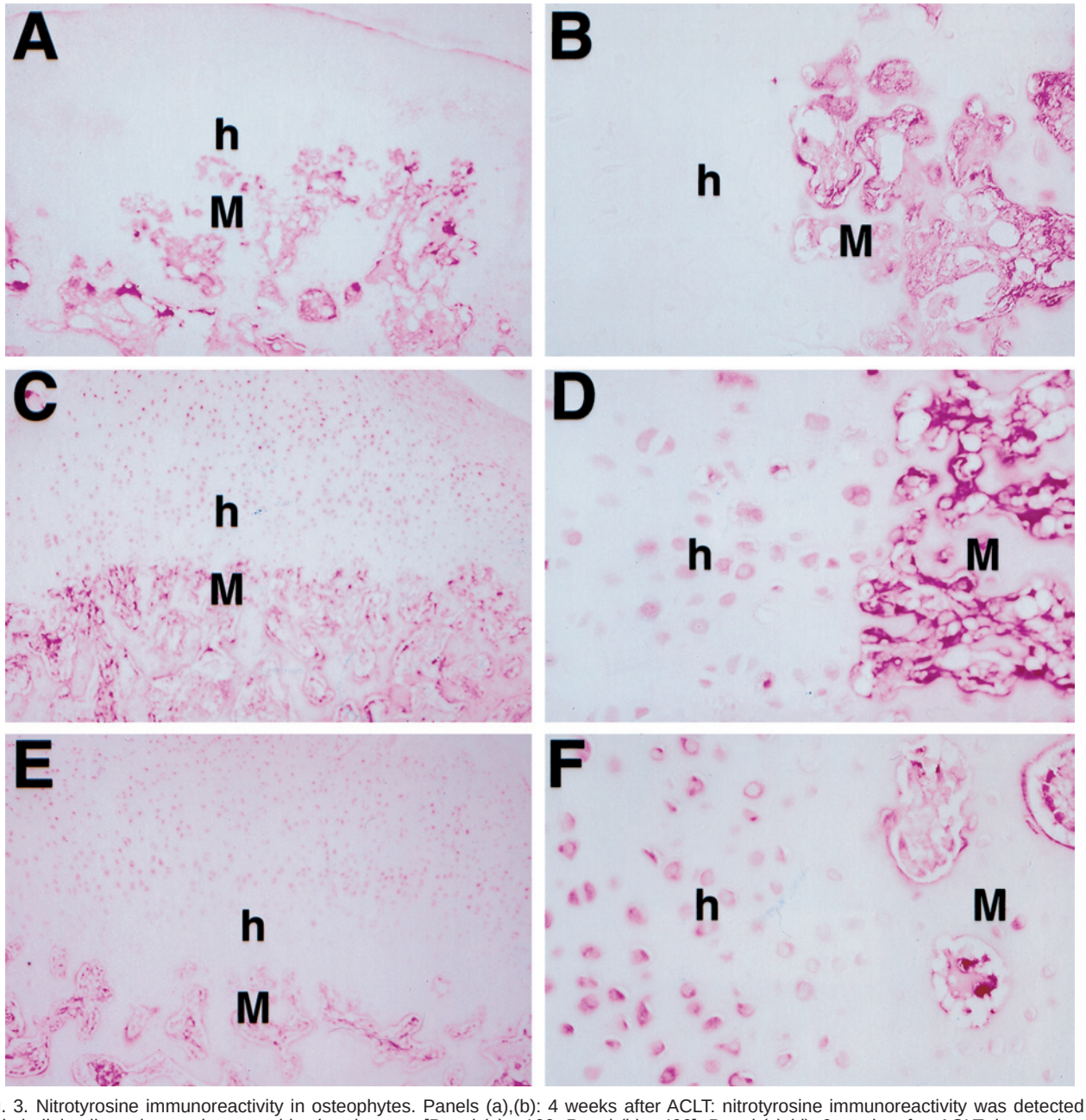

Fig. 3. Nitrotyrosine immunoreactivity in osteophytes. Panels (a),(b): 4 weeks after ACLT: nitrotyrosine immunoreactivity was detected in endothelial cells and some hypertrophic chondrocytes [Panel (a) ×100; Panel (b) ×400]. Panel (c),(d): 9 weeks after ACLT the number of nitrotyrosine positive cells was increased in the degenerative, hypertrophic and calcified zone [Panel (c) ×100; Panel (d) ×400]. Panel (e),(f): 12 weeks after ACLT the number of cells and intensity of nitrotyrosine immunoreactivity in the hypertrophic zone increases further. h=hypertrophic; M=mineralizing [Panel (e) ×100; Panel (f) ×400].

hypertrophy, vascular invasion and angiogenesis[24]. In normal joints only a small number of cells in the transition zone at the joint margin expressed VEGF [Fig. 2(a)]. Four weeks after ACLT new blood vessels had formed adjacent to hypertrophic chondrocytes. A small number of these hypertrophic cells were positive for VEGF [Fig. 2(b)]. Nine weeks after ACLT there were more blood vessels and now a large number of hypertrophic chondrocytes were expressing VEGF [Fig. 2(c)]. Some of the vascular cells were also positive for VEGF.

NITROTYROSINE FORMATION

Nitric oxide reacts with superoxide radical to form peroxynitrite. In tissue or biological fluids peroxynitrite leads to the nitration of aromatic amino acid residues and this may be a marker of peroxynitrite-mediated, NO-dependent damage *in vivo*[36]. Nitrotyrosine immunoreactivity was detected in vascular cells and some hypertrophic chondrocytes in the osteophytes 4 weeks after ACLT [Fig. 3(a),(b)]. After 9 and 12 weeks, a large number of nitrotyrosine

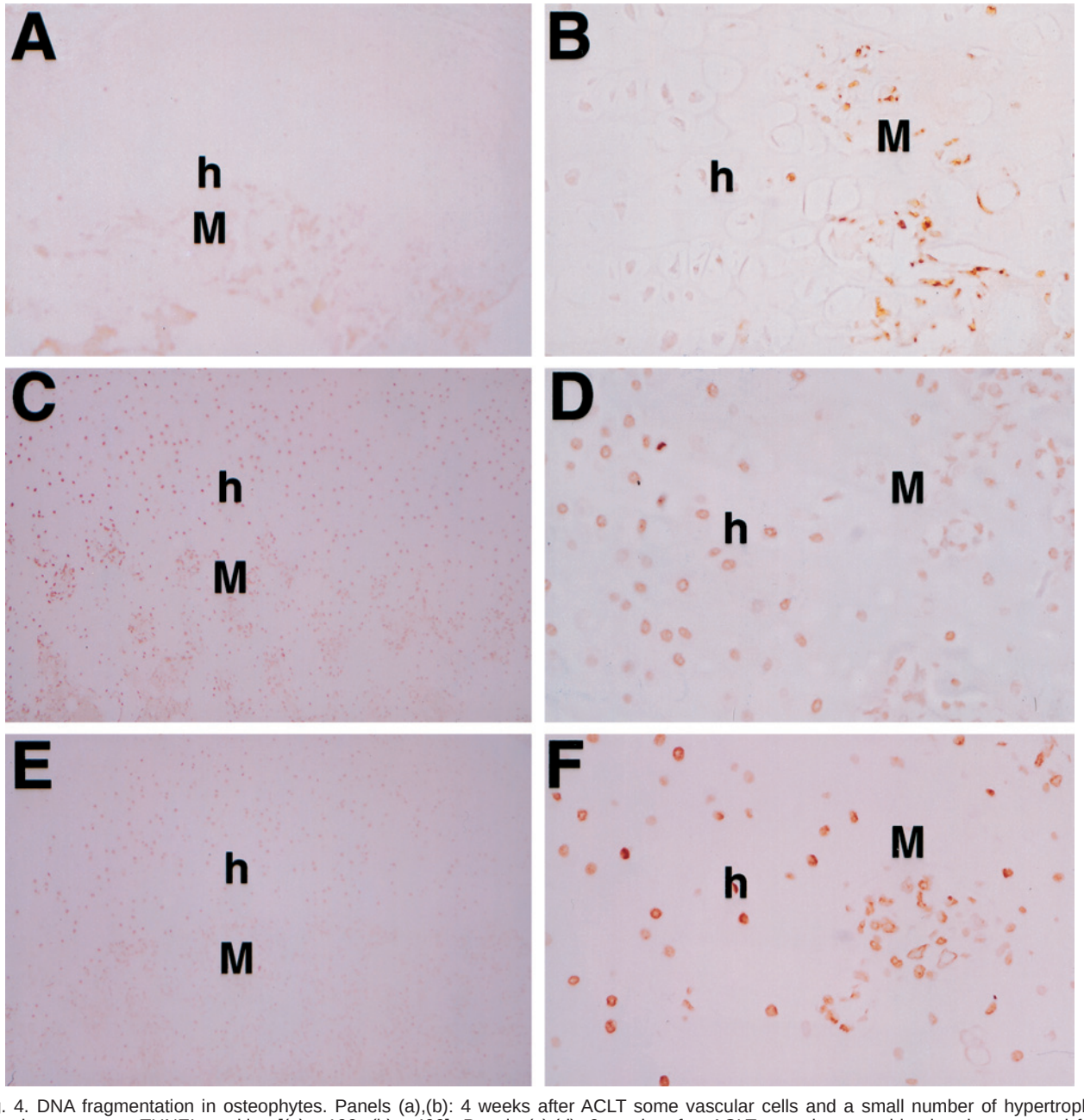

Fig. 4. DNA fragmentation in osteophytes. Panels (a),(b): 4 weeks after ACLT some vascular cells and a small number of hypertrophic chondrocytes were TUNEL positive [(a) ×100; (b) ×400]. Panels (c),(d): 9 weeks after ACLT most hypertrophic chondrocytes and few proliferative chondrocytes and many vascular cells were TUNEL positive [(c) ×100; (d) ×400]. Panels (e),(f): Many vascular cells and hypertrophic chondrocytes are TUNEL positive [(e) ×100; (f) ×400]. h=hypertrophic; M=mineralizing.

positive cells was present in the hypertrophic and in the calcified zone [Fig. 3(c)–(f)].

DNA FRAGMENTATION IN OSTEOPHYTES

Osteophytes that had developed 4 weeks after ACLT showed DNA fragmentation among some cells in blood vessels and hypertrophic chondrocytes close to the blood vessels [Fig. 4(a),(b)]. The proliferative zone, pre-hypertrophic zone and non-calcified hypertrophic zone showed only few apoptotic cells. Nine and 12 weeks after ACLT almost all cells in the hypertrophic zone and also some cells in the proliferative zone were undergoing apoptosis [Fig. 4(c)–(f)].

CO-LOCALIZATION OF DNA FRAGMENTATION AND NITROTYROSINE IMMUNOREACTIVITY

DNA fragmentation and nitrotyrosine immunoreactivity showed similar distribution in the osteophytes. Double staining revealed that many TUNEL-positive cells were also positive for nitrotyrosine during all phases of osteophyte development (Fig. 5).

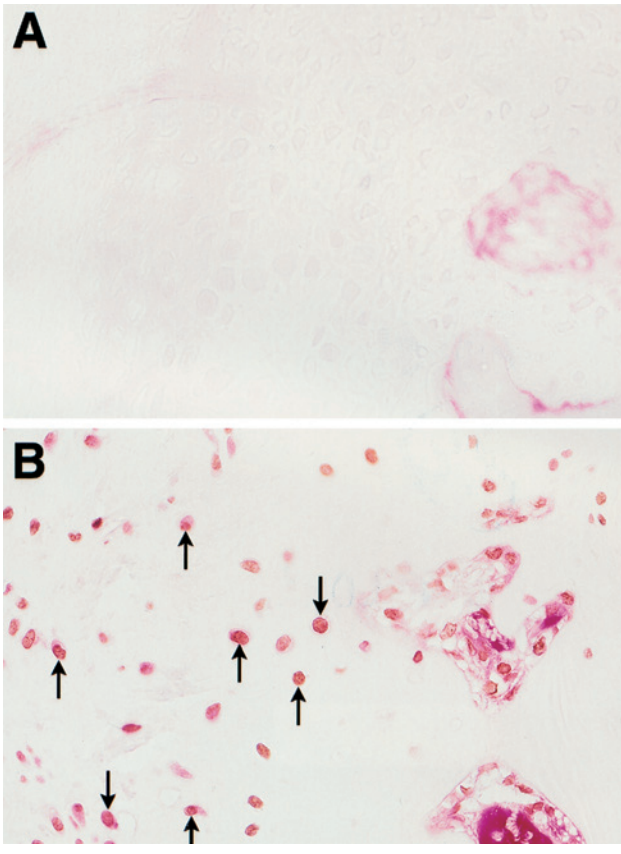

Fig. 5. Nitrotyrosine immunoreactivity and DNA fragmentation in osteophytes. Panel (a): 4 weeks after ACLT osteophyte stained with pre-immune rabbit IgG. Panel (b): Osteophyte 4 weeks after ACLT stained with nitrotyrosine and TUNEL (×400).

Discussion

Similarities in histologic appearance and regulatory pathways have been noted among the epiphyseal growth plate and osteophytes[22]. Chondrocyte hypertrophy, degeneration and vascular invasion with eventual replacement with new bone tissue are well characterized and mechanistically related events in growth plate[25,38]. The present study analysed whether VEGF and nitric oxide production can be detected in osteophytes and whether their expression is associated with chondrocyte death and matrix calcification.

The results show that hypertrophic chondrocytes in osteophytes express VEGF. In growth plate, hypertrophic chondrocytes produced VEGF and VEGF mediated capillary invasion is an essential signal that regulates chondrocyte differentiation and triggers cartilage remodeling[24]. VEGF inhibition expanded the layer of hypertrophic chondrocytes and inhibited or delayed apoptotic cell death in growth plate. The subchondral vascular system has a pivotal role in the regulation of endochondral ossification. Marginal osteophytes form when capillaries penetrate the subchondral bone and deep zone of articular cartilage. VEGF is thus likely to be an important regulator of angiogenesis during osteophyte development.

The so-called degeneration of chondrocytes in the hypertrophic zone is associated with DNA fragmentation. Cells with fragmented DNA were detected in the hypertrophic zone, while few cells were found in the proliferative zone. It is possible that at least some of the cells that are TUNEL positive are undergoing apoptosis. The formation of mineralizing matrix vesicles may thus also be a consequence of apoptosis in the osteophyte. Both chondrocyte-derived apoptotic bodies and matrix vesicles contain enzymatic activities that are required for calcium deposition[30]. Apoptosis in terminally differentiated growth plate chondrocytes has been reported by several laboratories[26,38]. The presence of TUNEL-positive cells in blood vessels may indicate that the involution of blood vessels occurs via apoptosis.

Nitrotyrosine immunoreactivity showed a similar distribution as apoptotic cells. In our previous studies, exogenous NO induced apoptotic cell death in cultured chondrocytes[34], and the presence of apoptotic chondrocytes correlated with NO production during experimental OA[33].

The present findings suggest that VEGF secreted from hypertrophic chondrocytes induced capillary invasion into the hypertrophic zone, calcified zone and subchondral zone from bone marrow. During the early phases (4 weeks after ACLT) endothelial cell derived NO may induce apoptosis in hypertrophic chondrocytes around blood vessels. At later phases (9 and 12 weeks), almost all degenerative and hypertrophic chondrocytes were nitrotyrosine-positive and these cells TUNEL-positive. Thus, NO production, may lead to chondrocyte apoptosis and both events contribute to the pathogenesis of cartilage degradation and osteophyte formation in the same joint. Inhibitors of NO synthesis were shown to reduce the progression of cartilage lesions, as well as the presence and size of osteophyte in experimental OA[39] and iNOS deficiency in mice with OA induced by intraarticular injection of collagenase was associated with reduced cartilage pathology and reduced osteophyte formation[40].

In conclusion, osteophyte formation during experimentally induced OA is associated with the expression of VEGF in chondrocytes. This provides a signal for vascular invasion of the cartilage and is followed by nitric oxide production, chondrocyte death and matrix calcification.

References

1. Bullough P. The pathology of osteoarthritis. In: Moskowitz R, Howell D, Goldberg V, Mankin H, Ed. Osteoarthritis. Philadelphia: W.B. Saunders 1992: 39–69.
2. Moskowitz RW, Goldberg VM. Osteophyte evolution: studies in an experimental partial meniscectomy model. J Rheumatol 1987;14 Spec No:116–8.
3. Moskowitz RW, Goldberg VM. Studies of osteophyte pathogenesis in experimentally induced osteoarthritis. J Rheumatol 1987;14:311–20.
4. Williams JA, Thonar EJ. Early osteophyte formation after chemically induced articular cartilage injury. Am J Sports Med 1989;17:7–15.
5. Brandt KD. Osteophytes in osteoarthritis. Clinical aspects. Osteoarthritis Cart 1999;7:334–5.
6. Marshall JL, Olsson SE. Instability of the knee. A long-term experimental study in dogs. J Bone Joint Surg [Am] 1971;53:1561–70.
7. Olsson SE, Marshall JL, Story E. Osteophytosis of the knee joint in the dog. A sign of instability. Acta Radiol (Suppl) 1972;319:165–7.
8. Danielsson L, Hernborg J. Clinical and roentgenologic study of knee joints with osteophytes. Clin Orthop 1970;69:302–12.

9. Shepstone L, Rogers J, Kirwan J, Silverman B. Distribution of distal femoral osteophytes in a human skeletal population. Ann Rheum Dis 2000;59:513–20.
10. Buckland-Wright JC, Lynch JA, Dave B. Early radiographic features in patients with anterior cruciate ligament rupture. Ann Rheum Dis 2000;59:641–6.
11. Marshall JL. Periarticular osteophytes. Initiation and formation in the knee of the dog. Clin Orthop 1969;62:37–47.
12. Buckland-Wright JC, Macfarlane DG, Lynch JA. Osteophytes in the osteoarthritic hand: their incidence, size, distribution, and progression. Ann Rheum Dis 1991;50:627–30.
13. Pelletier JP, Martel-Pelletier J. Protective effects of corticosteroids on cartilage lesions and osteophyte formation in the Pond-Nuki dog model of osteoarthritis. Arthritis Rheum 1989;32:181–93.
14. van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB. Differential effects of local application of BMP-2 or TGF-beta 1 on both articular cartilage composition and osteophyte formation. Osteoarthritis Cart 1998;6:306–17.
15. van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB. Osteoarthritis-like changes in the murine knee joint resulting from intra-articular transforming growth factor-beta injections. Osteoarthritis Cart 2000;8:25–33.
16. Okazaki K, Jingushi S, Ikenoue T, Urabe K, Sakai H, Ohtsuru A, *et al*. Expression of insulin-like growth factor I messenger ribonucleic acid in developing osteophytes in murine experimental osteoarthritis and in rats inoculated with growth hormone-secreting tumor. Endocrinology 1999;140:4821–30.
17. Middleton J, Arnott N, Walsh S, Beresford J. Osteoblasts and osteoclasts in adult human osteophyte tissue express the mRNAs for insulin-like growth factors I and II and the type 1 IGF receptor. Bone 1995;16:287–93.
18. Uchino M, Izumi T, Tominaga T, Wakita R, Minehara H, Sekiguchi M, Itoman M. Growth factor expression in the osteophytes of the human femoral head in osteoarthritis. Clin Orthop 2000;119–25.
19. Hughes SS, Hicks DG, O'Keefe RJ, Hurwitz SR, Crabb ID, Krasinskas AM, *et al*. Shared phenotypic expression of osteoblasts and chondrocytes in fracture callus. J Bone Miner Res 1995;10:533–44.
20. Sandberg M, Aro H, Multimaki P, Aho H, Vuorio E. In situ localization of collagen production by chondrocytes and osteoblasts in fracture callus. J Bone Joint Surg [Am] 1989;71:69–77.
21. Matyas JR, Sandell LJ, Adams ME. Gene expression of type II collagens in chondro-osteophytes in experimental osteoarthritis. Osteoarthritis Cart 1997;5: 99–105.
22. Aigner T, Dietz U, Stoss H, von der Mark K. Differential expression of collagen types I, II, III, and X in human osteophytes. Lab Invest 1995;73:236–43.
23. Eerola I, Salminen H, Lammi P, Lammi M, von der Mark K, Vuorio E, *et al*. Type X collagen, a natural component of mouse articular cartilage: association with growth, aging, and osteoarthritis. Arthritis Rheum 1998;41:1287–95.
24. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation [see comments]. Nat Med 1999;5:623–8.
25. Roach HI. New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. J Bone Miner Res 1997;12:795–805.
26. Hatori M, Klatte KJ, Teixeira CC, Shapiro IM. End labeling studies of fragmented DNA in the avian growth plate: evidence of apoptosis in terminally differentiated chondrocytes. J Bone Miner Res 1995;10:1960–8.
27. Zenmyo M, Komiya S, Kawabata R, Sasaguri Y, Inoue A, Morimatsu M. Morphological and biochemical evidence for apoptosis in the terminal hypertrophic chondrocytes of the growth plate. J Pathol 1996; 180:430–3.
28. Horton WE Jr, Feng L, Adams C. Chondrocyte apoptosis in development, aging and disease. Matrix Biol 1998;17:107–15.
29. Lee FY, Choi YW, Behrens FF, DeFouw DO, Einhorn TA. Programmed removal of chondrocytes during endochondral fracture healing. J Orthop Res 1998; 16:144–50.
30. Hashimoto S, Ochs RL, Rosen F, Quach J, McCabe G, Solan J, *et al*. Chondrocyte-derived apoptotic bodies and calcification of articular cartilage. Proc Natl Acad Sci USA 1998;95:3094–9.
31. Blanco FJ, Guitian R, Vazquez-Martul E, de Toro FJ, Galdo F. Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. Arthritis Rheum 1998;41:284–9.
32. Hashimoto S, Ochs RL, Komiya S, Lotz M. Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum 1998;41: 1632–8.
33. Hashimoto S, Takahashi K, Amiel D, Coutts RD, Lotz M. Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. Arthritis Rheum 1998;41:1266–74.
34. Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 1995;146:75–85.
35. Amin AR, Di Cesare PE, Vyas P, Attur M, Tzeng E, Billiar TR, *et al*. The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: evidence for up-regulated neuronal nitric oxide synthase. J Exp Med 1995;182: 2097–102.
36. Beckman JS. Peroxynitrite versus hydroxyl radical: the role of nitric oxide in superoxide-dependent cerebral injury. Ann NY Acad Sci 1994;738:69–75.
37. Yoshioka M, Shimizu C, Harwood FL, Coutts RD, Amiel D. The effects of hyaluronan during the development of osteoarthritis. Osteoarthritis Cart 1997;5:251–60.
38. Gibson G, Lin DL, Roque M. Apoptosis of terminally differentiated chondrocytes in culture. Exp Cell Res 1997;233:372–82.
39. Pelletier JP, Jovanovic DV, Lascau-Coman V, Fernandes JC, Manning PT, Connor JR, *et al*. Selective inhibition of inducible nitric oxide synthase reduces progression of experimental osteoarthritis in vivo: possible link with the reduction in chondrocyte apoptosis and caspase 3 level. Arthritis Rheum 2000; 43:1290–9.
40. van den Berg WB, van de Loo F, Joosten LA, Arntz OJ. Animal models of arthritis in NOS2-deficient mice. Osteoarthritis Cart 1999;7:413–5.