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Processes of Osteophyte Formation in Guinea Pigs with Spontaneous Osteoarthritis

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In this study, we investigated osteophyte formation processes in guinea pigs with spontaneous osteoarthritis, histochemically and immunohistochemically. Serial thin frontal sections of right knee joints were prepared from Hartley guinea pigs aged 1, 3, 5, 8, 12 and 18 months. The severity of osteoarthritis was evaluated by safranin-O staining, and the animals were classified into 3 groups: mild, moderate and severe. In addition, immunostaining was performed by using primary antibodies against the proliferating cell nuclear antigen (PCNA), type-I, -II and -III collagens, insulin-like growth factor 1 (IGF-1) and IGF-1 receptor. In the mild group, there was fibrous connective tissue continuous with the synovial membrane and covering the margins of the articular cartilage of the medial tibial condyle. This tissue contained spindle-shaped fibroblastic-like cells. These cells were positive for PCNA, type-I and -III collagens, IGF-1 and IGF-1 receptor. In the moderate group, the chondrocytes beneath the fibroblastic-like cell layer had proliferated and were clustered together. These chondrocytes were also positive for PCNA, type-I and -III collagens, IGF-1 and IGF-1 receptor. In the severe group, this marginal area had been replaced by type-II collagen-positive chondrophytes, which further changed to osteophytes due to the process of endochondral ossification. In guinea pigs, fibroblastic-like cells at the margins of the articular cartilage of the knee joints seemed to be totipotent immature mesenchymal cells. These cells may be the precursors of osteophytes, and IGF-1 appears to be involved in their formation.

Key words: guinea pig; insulin-like growth factor 1; osteoarthritis; osteophyte formation

The pathological changes in osteoarthritis include articular cartilage degeneration and proliferative changes, such as osteophyte formation and sclerosis of the subchondral bone. Sclerosis of the subchondral bone is regarded as a reactive phenomenon to excessive loading, but little is known about the mechanism of osteophyte formation (Jewell et al., 1998). It has been reported that acromegalic osteoarthropathy, which is categorized as secondary osteoarthritis, is characterized by hyperplasia of the articular cartilage and marked osteophyte formation (Bluestone et al., 1971; Jaffe, 1972; Johanson et al., 1983; Resnick, 1988). Insulin-like growth factor 1 (IGF-1) is thought to induce the characteristic pathology of this disease (Lieberman et al., 1992). IGF-1 has been demonstrated to promote mitosis and differentiation of articular chondrocytes, and to induce matrix synthesis (Ash and Francis, 1975; McQuillan et al., 1986; Luyten et al., 1988; Trippel et al., 1989). These facts suggest that IGF-1 may be generally involved in osteophyte formation in cases of osteoarthritis other than acromegalic osteoarthropathy, but this issue remains to be clarified.

Because osteophytes generally appear later than cartilage degeneration, the early processes of osteophyte development cannot be examined in human specimens, although observation of mature osteophytes is possible. We examined the processes of osteophyte formation in guinea

Abbreviations: IGF-1, insulin-like growth factor 1; PCNA, proliferating cell nuclear antigen

pigs, which show histological changes very similar to those in human osteoarthritis (Bendele et al., 1989; Okada et al., 1992; de Bri et al., 1996; Tokuda, 1997), using histological, histochemical and immunohistochemical techniques.

Materials and Methods

Preparation of experimental materials

One-, 3-, 5-, 8-, 12- and 18-month-old Hartley guinea pigs (Charles River Japan Inc., Tokyo, Japan) were sacrificed by intraperitoneal injection of pentobarbital sodium (150 mg/kg). We examined two 1-month-old (1 male, 1 female), three 3-month-old (1 male, 2 females), four 5-month-old (1 male, 3 females), four 8-month-old (2 males, 2 females), two 12-month-old (1 male, 1 female) and two 18-month-old (1 male, 1 female) guinea pigs. The right knee joints from these animals were fixed in 10% neutral formalin in a 90˚ flexed position for 3 days. Next, the knee joints were decalcified with 10% EDTA (pH 7.4) for 4 weeks at room temperature. The specimens were confirmed by soft X-ray that the specimens had been completely decalcified. After being dehydrated with ascending grades of ethanol and xylene, the specimens were embedded in paraffin. These specimens were cut into serial sections 7-μm thick each in the frontal plane passing through the midline between the medial and lateral menisci. These sections were subjected to hematoxylin and eosin, safranin-O and immunohistological staining.

Evaluation of osteoarthritis

By using safranin O-stained specimens from each animal, the degree of degeneration of the medial condyle of the tibia, in which cartilage degeneration develops earliest, was evaluated based on the histological-histochemical grading system

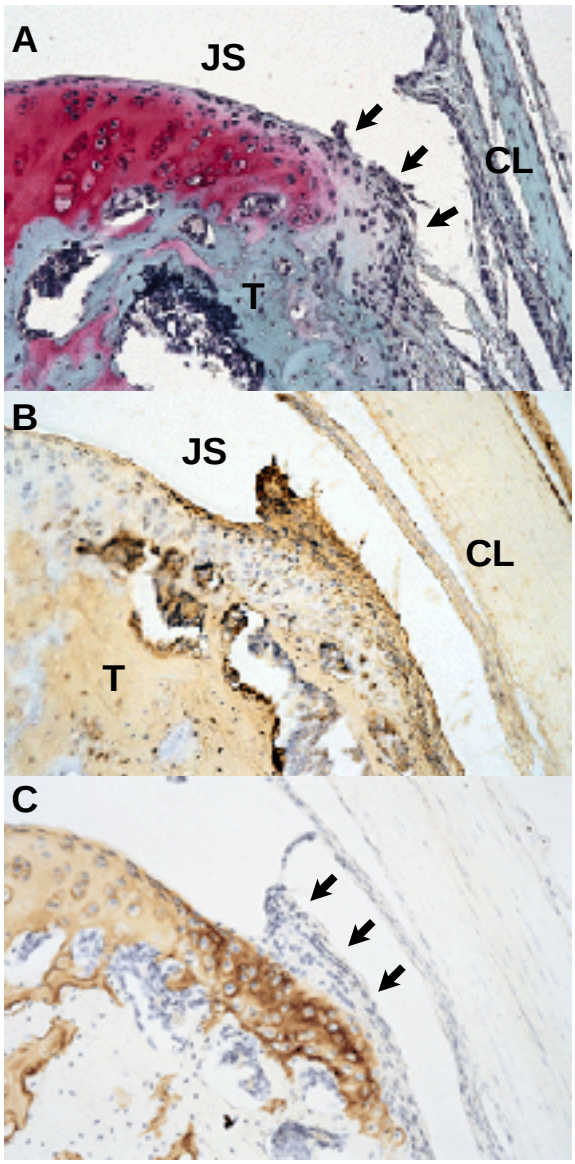
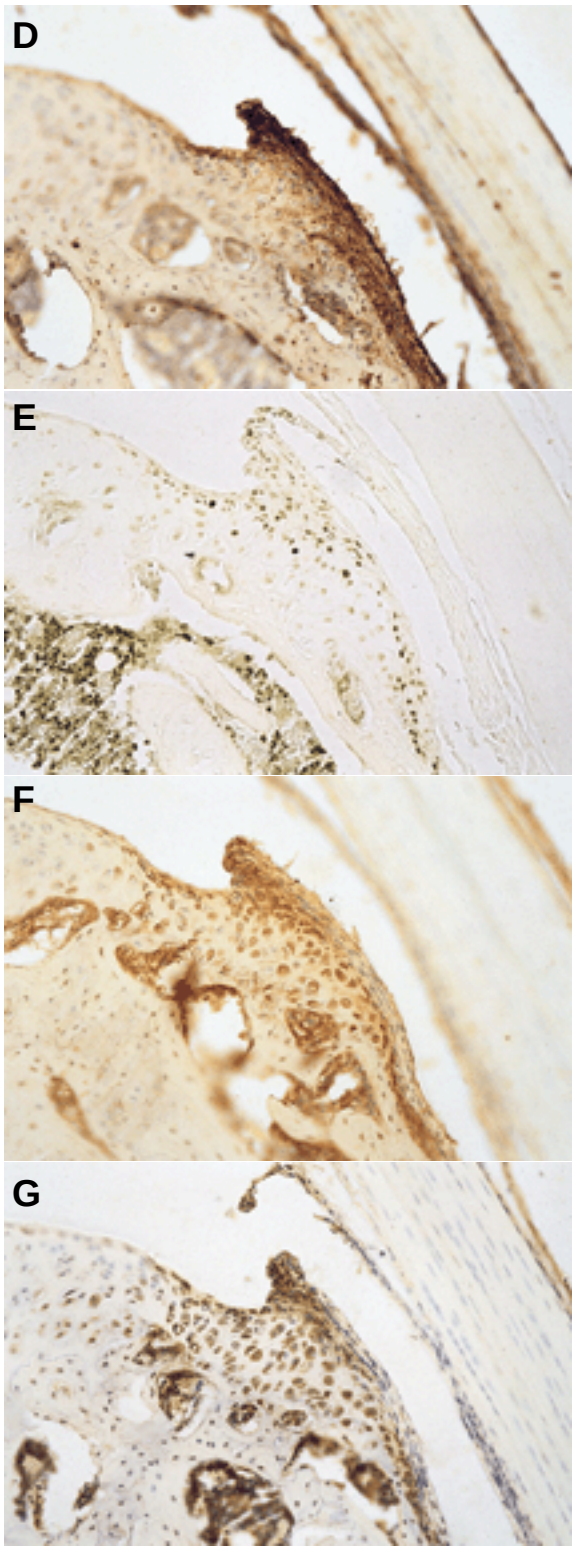


Fig. 1. Safranin-O staining (**A**) and immunohistological staining (**B**–**G**) show no chondrophyte formation in the medial margin of the tibial plateau in the mild group of 3-month-old animals without degenerative changes due to osteoarthritis. Areas in the fibrous connective tissue (arrows) negative for safranin-O staining (**A**) and for type-II collagen (**C**) are positive for type-I (**B**) and -III (**D**) collagens. The nuclei of fibroblastic-like cells in the fibrous connective tissue are positive for PCNA (**E**), IGF-1 (**F**) and IGF-1 receptor (**G**). CL, medial colateral ligament; JS, joint space; T, tibia. Original magnification, × 200.

[Figs. 1A–C on p. 132 and Figs. 1D–G on p. 133]



Figs. 1D–G. *Continued from the previous page.*

(Mankin et al., 1971). Because pannus formation was not observed in the degenerated cartilage of any of the guinea pigs at any age, this item was excluded. Thus, the degree was graded on a 6-tiered scale instead of a 7-tiered scale, with 13 as the total number of points. According to the cartilage degeneration score, animals were classified into the following 3 groups: the mild group (grades 0–2), the moderate group (grades 3–7) and the severe group (grades 8–13).

Immunohistological staining

Deparaffinized sections were reacted in solution containing bovine testicular hyaluronidase (Wako Kogyo Co. Ltd., Osaka, Japan; 2 mg/mL phosphate-buffered saline, pH 5.3, for 30 min at room temperature). For detection of type-II collagen, pronase treatment (Dako, Kyoto, Japan; 1 mg/mL phosphate-buffered saline, pH 7.3, for 30 min at room temperature) was added (Aigner et al., 1993). Protein treatment was not performed for detection of proliferating cell nuclear antigen (PCNA). For the secondary antibody, enzyme reagent and mixture of substrate and dye, Histofine streptavidin-biotin-PO (M) and (P) kits (Nichirei, Tokyo) were used, and staining was performed with the streptavidin-biotin method. Among primary antibodies, polyclonal anti-type-I collagen antibody (LSL, Tokyo; diluted at 1:500), monoclonal anti-PCNA antibody (Nichirei; non-diluted), monoclonal anti-IGF-1 antibody (Upstate Biotechnology, Lake Placid, NY; 100 µg/mL) and polyclonal anti-IGF-1 receptor antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1 µg/mL) were reacted in a moist chamber maintained at 4°C for about 24 h. Monoclonal anti-type-II and -type-III collagen antibodies (Fuji Chemical Industries, Takaoka, Japan; diluted at 1:1000 and 1:500, respectively) were reacted for 1 h at room temperature. Counterstaining was performed with 3% methylene green for anti-PCNA antibody and hematoxylin for

the other antibodies. It is reported that the structure of guinea pig IGF-1 is similar to that of human IGF-1 (LeRoith et al., 1993). In this study, we used anti-human IGF-1 antibody and anti-human IGF-1 receptor antibody for detection of IGF-1 and IGF-1 receptor of guinea pig.

Results

Based on the histological-histochemical grading system, five 1- to 5-month-old animals were classified as the mild group. The mean osteoarthritis grade was 0.60 ± 0.89. Seven 3- to 8-month-old animals were included in the moderate group, with a mean osteoarthritis grade of 5.14 ± 1.77. Five 8- to 18-month-old animals comprised the severe group, with a mean osteoarthritis grade of 9.40 ± 0.89.

Histopathological findings

Mild group (grades 0–2)

When articular cartilage of the medial condyle of the tibia in the mild group was examined, fibrous connective tissue continuous with the synovial membrane and covering the margins of the articular cartilage was observed at the synovium-cartilage junction in all animals. Fibrous connective tissue at this junction was not stained with safranin O. In the most superficial layer of the fibrous connective tissue at the junction, fibroblastic-like cells were present almost continuously from superficial cells of the synovial membrane. In a layer deeper than these fibroblastic-like cells, chondrocytes were observed, but these were not clustered. Osteophyte formation was not noted in any animal (Fig. 1A).

In the weight-bearing regions of the cartilage of animals showing osteoarthritis changes, a rougher structure of the superficial layer, slight proliferation of chondrocytes, and slightly decreased safranin-O staining intensity were noted.

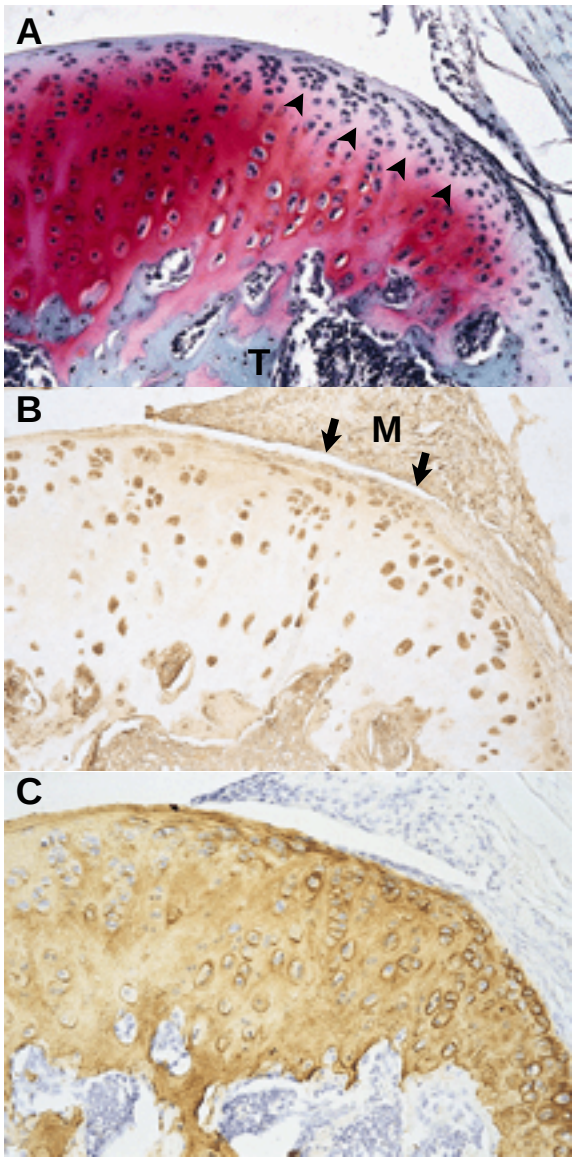
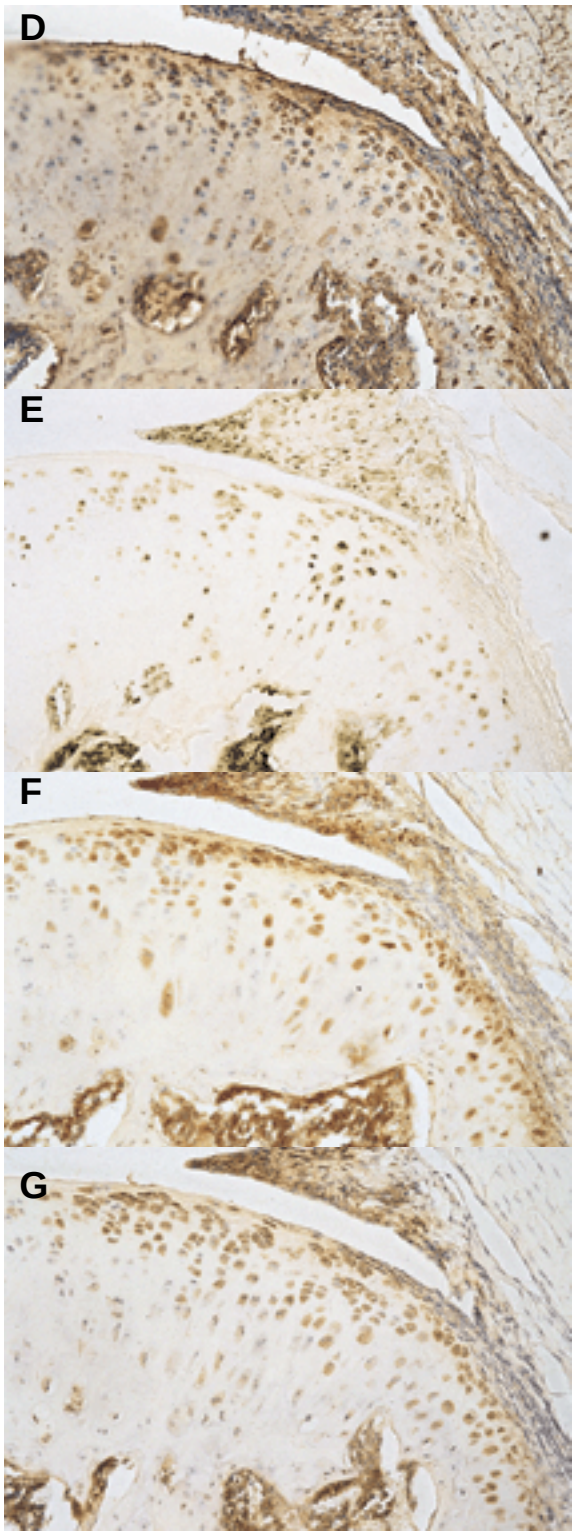


Fig. 2. Chondrocytes (arrowheads) cluster in a layer deeper than fibroblastic-like cells in the fibrous connective tissue (arrows) in the moderate group of 3-month-old animals, in which degenerative changes had progressed further (**A**, **B**). These chondrocytes, which are negative for type-I and -III collagens and PCNA in the mild group, are positive for all (**B**, **D**, **E** respectively) in the moderate group. This staining pattern is similar to that of fibroblastic-like cells in the fibrous connective tissue. Around these chondrocytes, cartilage matrix positive for type-II collagen is recognized (**C**). IGF-1 (**F**) and IGF-1 receptor (**G**) are identified in fibroblastic-like cells and chondrocytes seen in a layer deeper than the fibroblastic-like cells. M, medial meniscus; T, tibia. Original magnification, × 200. *[Figs. 2A–C on p. 134; Figs. 2D–G on p. 135]*



Figs. 2D–G. *Continued from the previous page.*

Moderate group (grades 3–7)

In the moderate group in which cartilage degeneration had progressed further, cells similar to chondrocytes had clustered in a layer below fibroblastic-like cells near the superficial layer of the margins of the articular cartilage. These cells had less cytoplasm. Chondrophyte formation was noted in 1 animal and osteophyte formation in another (Fig. 2A).

The weight-bearing regions of the cartilage showed fissures extending to the transitional zone and moderate to marked reduction in safranin-O staining intensity. The number of chondrocytes was decreased.

Severe group (grades 8–13)

A chondrophyte was identified in 1 animal (Fig. 3A) and osteophytes in 4 animals. Fibrous connective tissue of the synovium-cartilage junction and fibroblastic-like cells were noted around the osteophyte. Chondrocytes were recognized near the boundary between the osteophyte and weight-bearing regions of the cartilage, but had not clustered (Fig. 4A).

Fissures from the radial zone to the calcified zone and markedly reduced safranin-O staining intensity were noted in the weight-bearing regions of the cartilage. The number of chondrocytes was noticeably decreased.

Synovial membrane

The lining cells of the synovium consisted of 2 to 4 layers and proliferated more with osteoarthritis progression, but there were no findings suggestive of synovitis, such as villous formation or cell infiltration into sublining synovial tissue.

Immunohistological staining

Mild group

At the margins of the articular cartilage, the cytoplasm of fibroblastic-like cells in the fibrous connective tissue was positive for type-I (Fig. 1B) and -III (Fig. 1D) col

lagens. Moreover, the nuclei of these fibroblastic-like cells were positive for PCNA (Fig. 1E). Type-II collagen was not noted in either fibroblastic-like cells or fibrous connective tissue (Fig. 1C). IGF-1 (Fig. 1F) and IGF-1 receptor (Fig. 1G) showed almost the same distribution, being identified in the cytoplasm and areas around fibroblastic-like cells in the fibrous connective tissue. The results described above were obtained in all 5 animals. Chondrocytes noted in a layer deeper than fibroblastic-like cells were negative for PCNA in all but 1 animal, and the cartilage matrix surrounding the chondrocytes was positive for type-II collagen. These chondrocytes were positive for type-I collagen in 3 of 5 animals and for type-III collagen in 1 animal, but no animals were positive for PCNA and type-I and -III collagens at the same time.

In the weight-bearing regions of the cartilage, the cartilage matrix was almost homogeneously positive for type-II collagen. Chondrocytes in the tangential zone were positive for type-I collagen (2/5 animals), IGF-1 (5/5 animals) and IGF-1 receptor (5/5 animals), almost continuously from the articular margins. Chondrocytes in the tangential zone were positive for PCNA in only 1 animal.

Moderate group

Fibroblastic-like cells in the fibrous connective tissue were positive for type-I (Fig. 2B) and -III (Fig. 2D) collagens and PCNA (Fig. 2E) in all animals, as well as in the mild group. In chondrocytes clustering in the layer deeper than fibroblastic-like cells in the fibrous connective tissue, type-I and -III collagens and PCNA were identified in 4 animals. The extracellular matrix surrounding these chondrocytes was positive for type-II collagen (Fig. 2C). IGF-1 (Fig. 2F) and IGF-1 receptor (Fig. 2G) were identified in fibroblastic-like cells in the fibrous connective tissue and chondrocytes in the layer deeper than fibroblastic-like cells in all animals.

In the superficial layer of the weight-bearing regions of the cartilage, type-I (6/7 animals) and -III (5/7 animals) collagens were noted in the degenerated cartilage matrix. Staining for type-II collagen was not heterogeneous, and the degenerated cartilage matrix in the superficial layer of the weight-bearing regions showed stronger staining. Neither IGF-1 nor IGF-1 receptor was observed in the tangential zone or the deeper cartilage layer.

Severe group

At the articular margins, the bone component in the osteophytes was positive for type-I collagen (Fig. 4B) (5/5 animals). Fibroblastic-like cells in the fibrous connective tissue around

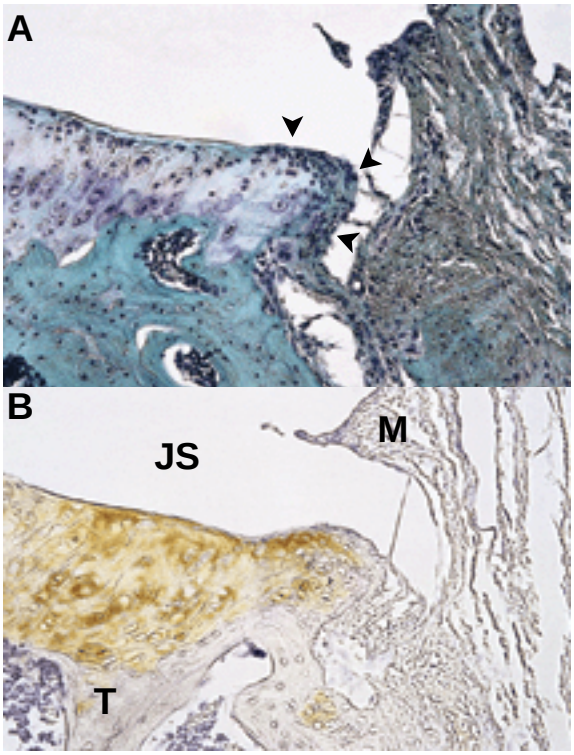


Fig. 3. Safranin-O staining (**A**) reveals a hump-like process (arrowheads) with a thick calcified zone from the medial articular margins of the tibial plateau in the severe group of 12-month-old animals with end-stage osteoarthritis. Chondrocytes are noted in the process, but not stained by safranin O. A distinctive tide mark is observed in a layer deeper than the cartilage layer (**A**). The calcified zone below the tide mark is positive for type-II collagen (**B**), suggesting that the hump-like process is chondrophyte. JS, joint space; M, medial meniscus; T, tibia. Original magnification, × 200.

the osteophyte were positive for type-I and -III collagens (Fig. 4D) and slightly positive for PCNA (Fig.4E) (5/5 animals). Type-II collagen was not observed in the fibrous connective tissue (Fig. 4C). IGF-1 (Fig. 4F) and IGF-1 receptor (Fig.4G) were noted in the fibrous connective tissue of all animals.

In the weight-bearing regions, the staining intensity for type-II collagen in the cartilage matrix tended to decrease with osteoarthritis progression. The staining intensities for type-I and -III collagens were increased mainly in the superficial layer of the degenerated cartilage compared with those in the mild and moderate groups. No PCNA-positive cells were detected in the weight-bearing regions of the cartilage. The superficial matrix of the degenerated cartilage was positive for IGF-1 and IGF-1 receptor (5/5 animals).

Synovial membrane

IGF-1 and IGF-1 receptor showed the same staining pattern, a comparatively homogeneous distribution in the cytoplasm of lining cells of the synovial membrane. There was no intergroup difference in this staining pattern.

Discussion

A study in human knee joints by Allard et al. (1990) has suggested the presence of fibrous connective tissue that is connected to the synovial membrane, is not stained with safranin O, and differs from articular cartilage at the margins. Using a rabbit model for experimental osteoarthritis, Moskowitz and Goldberg (1987) have demonstrated that cells with a very high regenerative capacity are present in the junction between the synovial membrane and perichondrium or between the synovial membrane and periosteum at the margin of the articular cartilage, and these cells are involved in osteophyte formation. Telhag and Lindberg (1972) also reported similar results. Moreover, an immunohistological study has demonstrated that fibroblastic-like cells in the fibrous connective tissue produce a connective tissue matrix mainly composed of type-I and -III collagens (Aigner et al., 1995). The presence of type-I and -III collagens was confirmed in totipotent immature mesenchymal cells of the cartilagenous anlage during the embryonic period (Linsenmayer et al., 1973; von der Mark et al., 1976; von der Mark and von der Mark, 1977). In this study, we confirmed the presence of fibroblastic-like cells that were embedded in the fibrous connective tissue and were positive for type-I and -III collagens, but were negative for type-II collagen at the cartilagenous margins of the knee joint in guinea pigs. It was also demonstrated that these cells were positive for PCNA as well, indicating that they have a high mitotic activity. Consequently, fibroblastic-like cells at the margins of the articular cartilage seemed to be totipotent immature mesenchymal cells based on the features of the staining pattern for collagens and on having differentiating potential.

Fibrous connective tissue was present at the articular margins, and fibroblastic-like cells presumed to be totipotent immature mesenchymal cells were identified in this connective tissue in all animals irrespective of the degree of osteoarthritis. Mitotic activity of chondrocytes observed in the layer deeper than these fibroblastic-like cells varied according to the degree of osteoarthritis. In the mild group, no mitotic activity of the chondrocytes at the margins was enhanced. In the moderate group, however, chondrocytes with an enhanced mitotic activity had proliferated and clustered. Around these chondrocytes, a cartilage matrix positive for type-II collagen was identified. In the severe group, in which osteoarthritis had progressed further, a hump-like process with a thick calcified zone, which was considered to be the chondrophyte, was observed. Furthermore, this area had been substituted by bone tissue positive for type-I collagen. These findings suggest that the mechanism of osteophyte formation is mediated by endochondral ossification.

IGF-1 and IGF-1 receptor showed almost the same distribution in all animals. This result demonstrates that chondrocytes with an IGF-1 secretory activity and those with IGF-1 receptor exist in the joints. In particular, totipotent immature mesenchymal cells at the margins of the

articular cartilage were positive for IGF-1 and IGF-1 receptor in all animals with osteoarthritis in each grade. In the moderate group, in which articular cartilage degeneration had progressed further, the presence of IGF-1 and IGF-1 receptor was noted not only in fibroblastic-like cells, but also in chondrocytes clustering in a layer deeper than fibroblastic-like cells. In contrast to chondrocytes in the weight-bearing regions, these chondrocytes proved to have a very high mitotic activity in this study. Therefore, it can be speculated that chondrocytes presumed to be differentiated from totipotent immature mesenchymal cells may have produced a cartilage matrix by the autocrine mechanism via IGF-1. A previous report has demonstrated that more IGF-1 is present in human osteoarthritis cartilage and synovial fluid than in normal cartilage and synovial fluid, and suggested that this IGF-1 originated from the synovial membrane (Schneiderman et al., 1995). However, this study could not demonstrate that IGF-1 secreted by totipotent immature mesenchymal cells directly participated in the differentiation to chondrocytes. Some studies have indicated the close involvement of TGF-beta 1 in experimental osteophyte formation (van Beuningen et al., 1994; van den Berg, 1995), and speculated that TGF-beta 1 has a facilitatory effect on differentiation of totipotent immature mesenchymal cells. Furthermore, other growth factors are also thought to interact in a complicated manner (Trippel, 1995).

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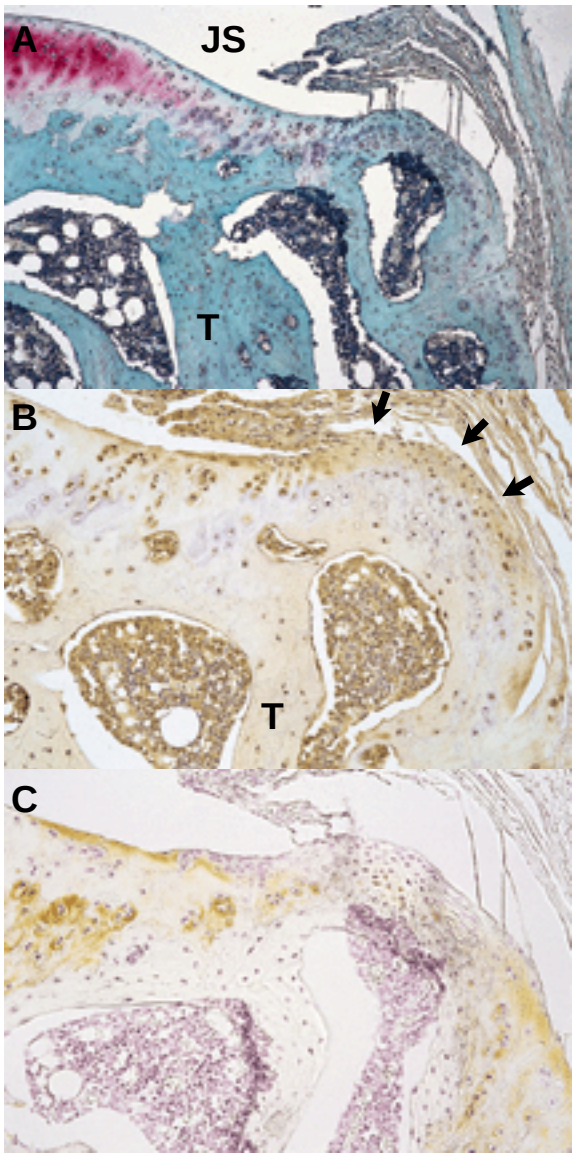
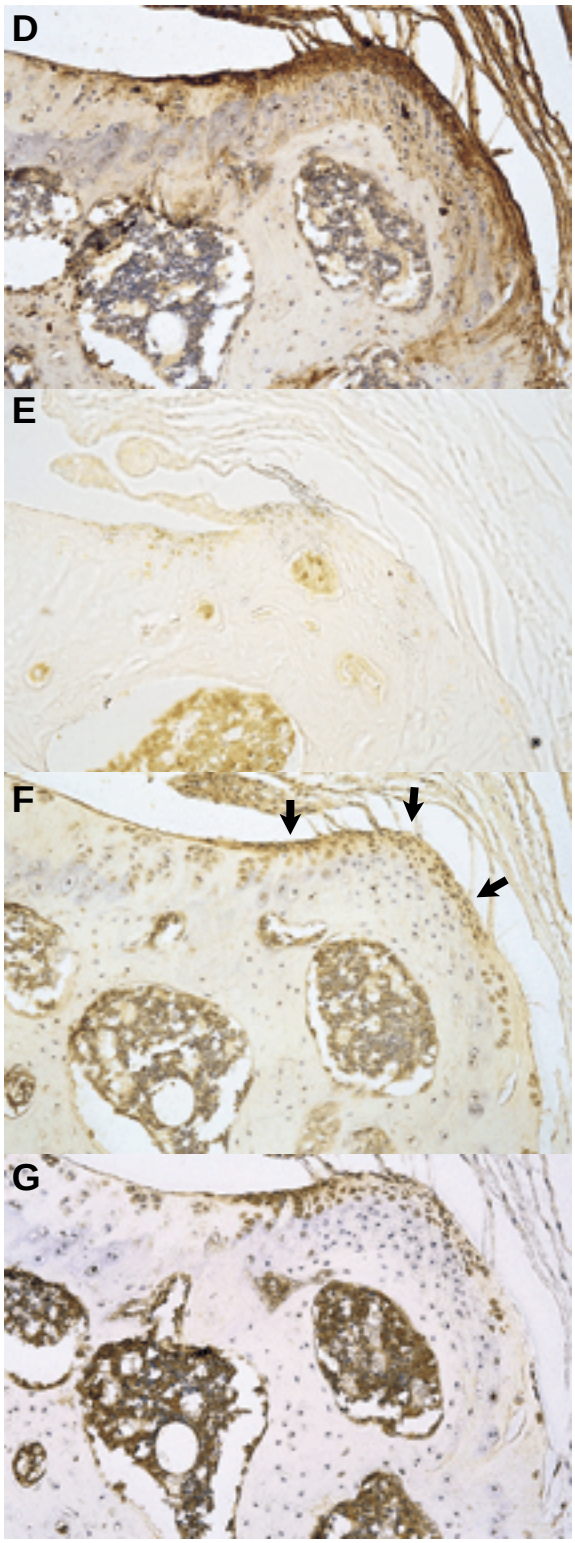


Fig. 4. Safranin-O (**A**) staining discloses the obvious formation of an osteophyte protruding from the articular margin in the severe group of 18-month-old animals with end-stage osteoarthritis. The osteophyte consists of type-I collagen (**B**), and hardly any chondrocytes and cartilage matrix positive for type-II collagen are observed (**C**). Fibrous connective tissue (arrows) is noted around the osteophyte, and IGF-1 (**F**) and IGF-1 receptor (**G**) are identified in the fibrous connective tissue. Fibroblastic-like cells in the fibrous connective tissue are positive for both type-I (**B**) and -III (**D**) collagens, and slightly positive for PCNA (**E**). JS, joint space; T, tibia. Original magnification: A, × 100; B–G, × 200.

[Figs. 4A–C on p. 138; Figs. 4D–G on p. 139]



Figs. 4D–G. *Continued from the previous page.*

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