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The Cholinergic System Can Be of Unexpected Importance in Osteoarthritis

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1. Introduction

The main belief is that joints such as the knee and ankle joints are not innervated by nerves with a cholinergic function. That includes the assumption that these joints are not innervated by the vagus nerve (van Maanen et al., 2009a, see also Grimsholm et al., 2008). Accordingly, there is actually no morphologic proof of a cholinergic innervation of the knee joint, nor of the ankle joint. Despite this fact, it is shown that electrical and pharmacological stimulation of the vagus nerve has a diminishing effect on carragenan-induced paw inflammation in rats (Borovikova et al., 2000a) and that interference with the effects of the vagus nerve leads to effects on the knee joint arthritis as seen experimentally (van Maanen et al., 2009b). There are also other findings which show the potential effects that interference with vagal effects has on joint inflammation. These will be discussed below.

It is actually strange that interference with cholinergic effects, as via manipulations of the vagus nerve, has effects in knee joint inflamed synovium without presence of a vagal nerve innervation. One possibility is that the effects are indirect, via an occurrence of vagal effects on other sites such as the spleen (Huston et al., 2006, see also van Maanen et al., 2009a). However, another possibility is that there is a non-neuronal production of acetylcholine (ACh) within the synovial tissue itself. This has actually been shown to be the case (Grimsholm et al., 2008) (see further in paragraph 3).

2. Non-neuronal ACh production – General aspects

It is nowadays known that there is a production of ACh in non-neuronal cells. The information has especially emerged via studies on expressions of the ACh-producing enzyme choline acetyltransferase (ChAT), but new knowledge on this topic has also become evident via studies of expressions of vesicular acetylcholine transporter (VAChT), carnitine acetyltransferase (CarAT), and the high-affinity choline transporter (CHT1). It is likely that the ACh produced in non-neuronal cells is released directly after synthesis, in contrast to the nerve-related ACh which is released via exocytosis.

Cell types for which ACh production has been shown are cells in the airways (Wessler et al., 2003, 2007), the keratinocytes of the skin (Grando et al., 1993, 2006), cells of the intestinal epithelium (Klapproth et al., 1997; Ratcliffe et al., 1998; Jönsson et al., 2007), cells of the urinary bladder wall (Lips et al., 2007; Yoshida et al., 2008), cells in blood vessel walls (Kirkpatrick et al., 2003; Lips et al., 2003) and certain cancer cells (Song et al., 2003, 2008;

Paleari et al., 2008). A further celltype for which there is evidence of ACh production is the tenocyte of human patellar (Danielson et al., 2006, 2007) and Achilles (Bjur et al., 2008) tenocytes. It was hereby found that the evidence was much stronger for chronic painful (tendinosis) tendons than normal pain-free tendons (Danielson et al., 2006, 2007; Bjur et al., 2008). Existence of a non-neuronal cholinergic system has also recently been shown for osteoblast-like cells (En-Nosse et al., 2009) and hepatocytes (Delbro et al., 2011).

Of special interest with respect to what will be discussed below, is the fact that inflammatory cells (Kawashima & Fuji, 2004) and fibroblasts (Fisher et al., 1993; Lips et al., 2003) show production of ACh. It should here be remembered that the tenocytes of human tendons in principle have fibroblast-like appearances.

3. Non-neuronal ACh production in synovium

It has previously been unclear as to whether there is a non-neuronal cholinergic system in synovial tissues. However, studies performed during recent years have provided evidence of ACh production in the synovial tissue of the human knee joint (Grimsholm et al., 2008). That was shown both via immunohistochemistry and in situ hybridization and was related to findings of ChAT expression in mononuclear-like as well as fibroblast-like cells (Grimsholm et al., 2008). The findings were shown both for synovial tissue of patients with rheumatoid arthritis (RA) as well as patients with osteoarthritis (OA). The occurrence of ChAT expression in mononuclear-like cells in OA synovium is shown below (Fig 1).

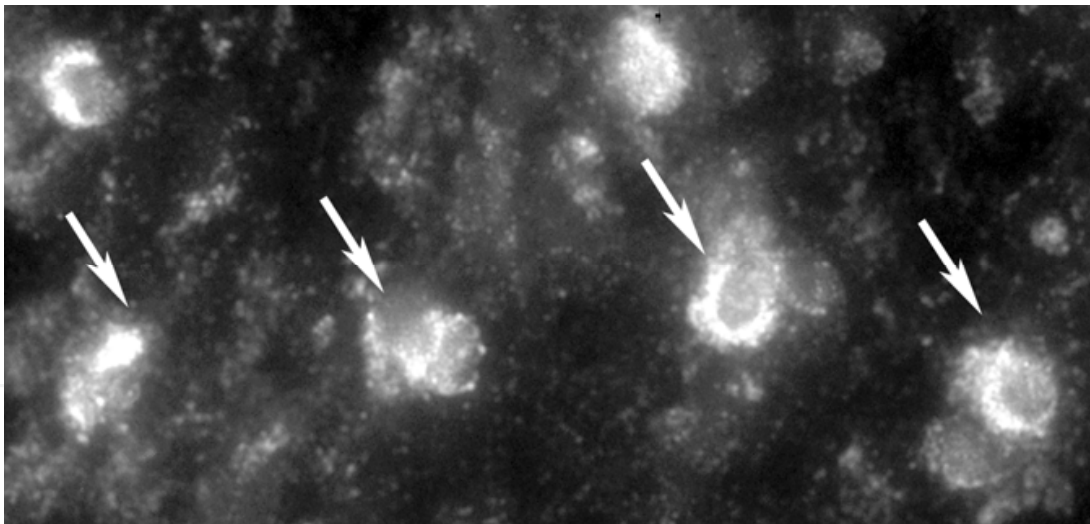


Fig. 1. Figure showing the expression of ChAT in mononuclear-like cells in the OA synovial tissue. Some of the immunoreactive cells are indicated with arrows.

4. Functions of non-neuronally produced ACh

The effects of the non-neuronal cholinergic system include functions on growth/differentiation and secretion and barrier functions (c.f. Wessler & Kirkpatrick, 2001, 2008). ACh has e.g. well-known effects on angiogenesis (Jacobi et al., 2002; Cooke et al., 2007). It is also known that an increased cell proliferation occurs in response to cholinergic stimulation (Mayerhofer & Fritz, 2002; Metzen et al., 2003; Oben et al., 2003). That includes proliferative effects on human fibroblasts (Matthiesen et al., 2006). Interestingly, it is also

frequently emphasized that ACh not only is produced in immune cells but that it also has effects on these cells, ACh hereby modulating the activity of the immune cells via auto- and paracrine loops (Kawashima & Fuji, 2003, 2008). The findings that ACh hereby has anti-inflammatory effects, findings which will be considered below, are especially interesting, but it is also possible that acute ACh stimulation can lead to proinflammatory effects (cf. Kawashima & Fuji, 2003).

5. Cholinergic anti-inflammatory pathway

A newly recognized concept is the "cholinergic anti-inflammatory pathway" (Borovikova et al., 2000b; Pavlov & Tracey, 2005; Tracey 2007). It is related to the occurrence of immunomodulatory effects of ACh released from cholinergic nerves. For example, as is commented on above, there occurs a suppression of the inflammation in the carrageenan paw edema in the rat in response to activation of this anti-inflammatory pathway via pharmacologic or electrical stimulation of the vagus nerve (Borovikova et al., 2000a). There is furthermore an attenuation in macrophage activation in response to electrical stimulation of the vagus nerve (de Jonge et al., 2005) and stimulation of the vagus nerve does on the whole improve survival in animal models of inflammation (e.g. Bernik et al., 2002). Neural inputs to immune cells can control cytokine production (Tracey, 2007). Concerning joints, there is evidence of a role of the cholinergic anti-inflammatory pathway in the murine CIA model of RA (van Maanen et al., 2010). Studies on the synovium in RA do nevertheless suggest that the cholinergic anti-inflammatory pathway might be suppressed in this condition (Goldstein et al., 2007).

It can be asked as to whether ACh originating from non-neuronal cells can be involved in the anti-inflammatory pathway. This can actually be the case. It is thus possible that neuronally released ACh triggers the release of ACh from these non-neuronal cells (Wessler & Kirkpatrick., 2008) and that effects via the non-neuronal cholinergic system even can occur independently of actions via cholinergic nerves (Kawashima & Fuji, 2003). Further evidence is the finding that ACh-induced modulation of immune functions in peripheral leukocytes occurs independently of neuronal innervation (Neumann et al., 2007). The non-neuronal ACh production in synovial tissue might therefore be of importance in the regulation of the processes that occur in this tissue in various forms of arthritis, including OA.

6. Involvement of the $\alpha 7$ nAChR in anti-inflammatory effects

The nicotinic acetylcholine receptor AChR $\alpha 7$ ($\alpha 7$ nAChR) is considered to be important in the cholinergic anti-inflammatory pathway (Wang et al., 2003; Kawashima & Fuji, 2008). The $\alpha 7$ nAChR is thus shown to contribute to anti-inflammatory effects of ACh in several models (Tracey, 2002; Ulloa, 2005; de Jonge & Ulloa, 2007). $\alpha 7$ nAChR agonists are furthermore shown to suppress the production of TNF alpha, IL-1, IL-6 and IL-8 and various other cytokines in macrophages after challenge with lipopolysaccharide (Borovikova et al., 2000a; Wang et al., 2003). An $\alpha 7$ nAChR agonist is also shown to decrease the production of IL-6 by IL-1 stimulated fibroblast-like synoviocytes (Waldburger et al., 2008). The results of still other studies show that specific $\alpha 7$ nAChR agonists can reduce TNFalpha-induced IL-6 as well as IL-8 production by fibroblast-like synoviocytes (van Maanen et al., 2009c).

7. Current study: Expression of $\alpha 7$ nAChR in osteoarthritis

7.1 Introduction

Except for effects in the autonomic nervous system, ACh is reported to have proliferative and growth-promoting effects, effects in cancer progression and anti-inflammatory effects. As ACh has anti-inflammatory effects and effects in relation to growth/proliferation, it is of interest to consider its importance in arthritic processes. The findings that mononuclear- as well as fibroblast-like cells in the synovium of the knee joints of patients with RA as well as OA show ChAT, favouring ACh production, is therefore of interest (Grimsholm et al., 2008). The receptor through which the inflammatory-mediating effects of ACh is reported to be mainly mediated is the $\alpha 7$ nAChR (Kawashima & Fuji., 2008). It is therefore of interest to note that expression of $\alpha 7$ nAChR has been shown for the synovial tissue of patients with RA and psoriatic arthritis (van Maanen et al., 2009a; Westman et al., 2009). The receptor was also to some extent noted for the synovium of healthy individuals (Westman et al., 2009). In studies on synovial tissue from 3 patients with OA, as well 3 patients with RA, it was shown that the $\alpha 7$ nAChR was expressed in the synovial intimal lining (Waldburger et al., 2008). The details of receptor expression at the tissue level concerning OA has not been further studied. More information is therefore welcome concerning the situation in OA. Therefore, the expression pattern of the $\alpha 7$ nAChR in the knee synovial joint of patients with OA was examined in the present study.

7.2 Materials & methods

7.2.1 Patient material

Synovial biopsies were collected from the knee joint of six patients with OA. Four of these were females (range 58-81 years; mean age 68 years) and two were males (50 and 62 years of age). The biopsies were obtained during prosthesis operations. They thus corresponded to samples of cases with advanced and long-lasting OA. The OA patients fulfilled the criteria of Altman and co-writers (Altman et al., 1986). All study protocols were approved by the Regional Ethical Review Board in Umeå (EPN) (project nr. 05-016M). The experiments were conducted according to the principles expressed in the Declaration of Helsinki. Informed consent was obtained from all individuals.

7.2.2 Fixation and sectioning

Directly after the surgical procedures, the tissue samples were transported to the laboratory. They were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.0) at 4°C for 24 h and were then washed in Tyrode's solution (pH 7.2) containing 10% (w/v) sucrose for 24 h, mounted on thin cardboard with OCT embedding medium (Miles Laboratories, Naperville, IL, USA) and frozen in propane chilled by liquid N₂. A series of 7 μ m thick sections were cut on a cryostat and mounted on slides coated with chrome-alum gelatine for immunohistochemistry. Some of the sections were stained with haematoxylin-eosin (htx-eosin) for delineating tissue morphology.

7.2.3 Immunofluorescence staining

The sections were mounted in Vectashield hard set microscopy mounting medium (Dakopatts, Denmark). The immunohistochemical procedures were in principle as previously described (Bjur et al., 2008). In order to enhance specific immunoreactions,

treatment with KMnO_4 was applied in accordance with developed procedures in the laboratory (Hansson & Forsgren., 1995).

The sections were incubated for 20 min in a 1% solution of Triton X-100 (Kebo lab, Stockholm) in 0.01 M phosphate buffer saline (PBS), pH 7.2, containing 0.1% sodium azide as preservative, and thereafter rinsed in PBS three times, 5 min each time. The sections were then incubated with 5% normal donkey serum in PBS. The sections were thereafter incubated with the primary antibody, diluted in PBS, in a humid environment. Incubation was performed for 60 min at 37°C. After incubation with specific antiserum, and three 5 min washes in PBS, another incubation in normal donkey serum followed, after which the sections were incubated with secondary antibody. As secondary antibody, a FITC-conjugated donkey anti-goat IgG (Jackson Immunoresearch, West Grove, PA), diluted 1:100, was used. Incubation with secondary antibody was performed for 30 min at 37°C. The sections were thereafter washed in PBS and then mounted in Vectashield Mounting Medium (H-1000) (Vector Laboratories, Burlingame, CA, USA). Examination was carried out in a Zeiss Axioscope 2 plus microscope equipped with an Olympus DP70 digital camera. The primary antibody was an antibody against the nicotinic acetylcholine receptor $\text{AChR}\alpha 7$ ($\alpha 7\text{nAChR}$). This antibody is an affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of $\alpha 7\text{nAChR}$ of human origin (Santa Cruz Biotechnology; sc-1447, dilution used 1:100). The outcome of immunostaining using the used protocol, including the currently used secondary antiserum, with primary antibody being substituted by PBS or normal serum, has been previously evaluated for human tissue (Danielson et al 2006a; Bjur et al., 2008) (control stainings). Sections of fixed rabbit muscle/inflammatory tissue were furthermore processed in parallel for control purposes, the same procedures as used here for demonstration of $\alpha 7\text{nAChR}$ immunoreactions in the synovial tissue being used. It was hereby found that the inflammatory cells in the muscle inflammation (myositis) showed distinct $\alpha 7\text{nAChR}$ immunoreactions, whilst the muscle tissue did not (not shown). Occurrence of $\alpha 7\text{nAChR}$ immunoreactions for inflammatory cells in muscle inflammation (myositis) is a well-known fact (Leite et al., 2010).

7.3 Results

Mononuclear-like and fibroblast-like cells occurred to varying extents in the synovial samples. They mainly lay scattered in the tissue. The degree of the inflammatory response varied greatly.

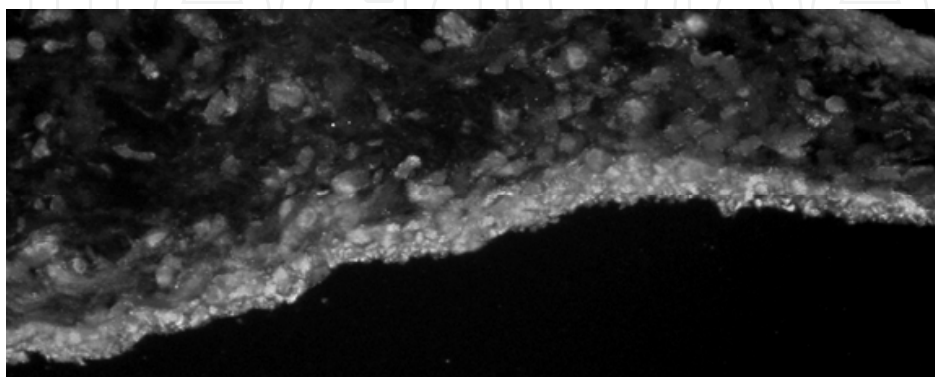


Fig. 2. Figure showing existence of marked $\alpha 7\text{nAChR}$ immunoreactions in the synovial lining layer.

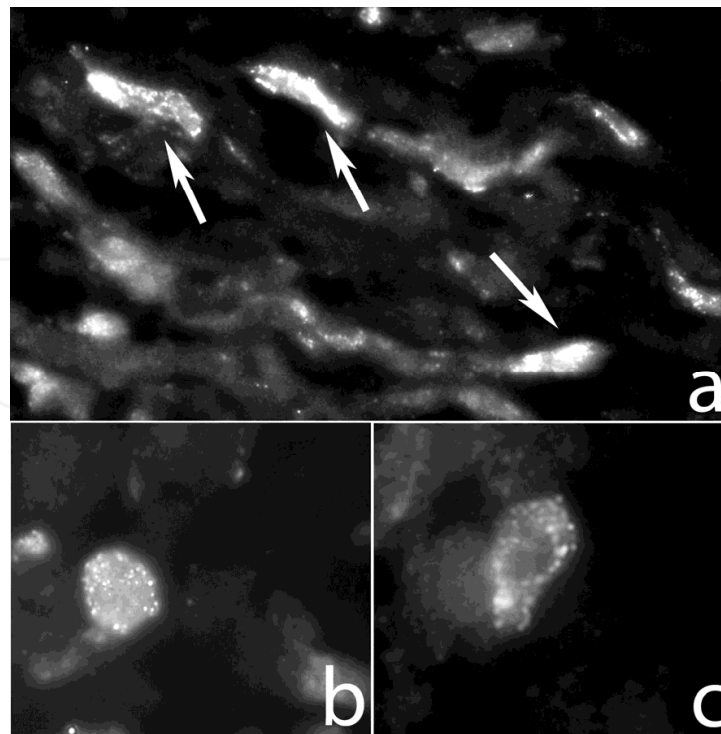


Fig. 3. Figure showing presence of $\alpha 7$ nAChR immunoreactions in fibroblast-like (a) and mononuclear-like (b,c) cells in the synovial tissue. Arrows at fibroblast-like cells in (a).

Marked $\alpha 7$ nAChR immunoreactions (IR) were seen in the synovial lining layer (Fig 2). $\alpha 7$ nAChR IR were also seen for mononuclear-like and fibroblast-like cells (Fig 3).

7.4 Discussion

The observations show that there indeed is immunolabelling for the $\alpha 7$ nAChR in the synovial tissue of patients with advanced OA. The findings are in line with recent findings of $\alpha 7$ nAChR immunoreactions in the synovial lining layer for RA patients (van Maanen et al., 2009a) and similar findings made in another recent study on patients with RA and psoriatic arthritis (Westman et al., 2009). The observations are also in line with the findings of scattered cells showing fibroblast-like and mononuclear-like appearances exhibiting $\alpha 7$ nAChR immunoreactions in RA and psoriatic arthritis (Westman et al., 2009). Furthermore, cultured RA fibroblast-like synoviocytes have been found to express $\alpha 7$ nAChR (van Maanen et al., 2009a).

OA synovial intimal lining as well as cultured fibroblast-like synoviocytes obtained from synovial tissue from 3 OA patients express the $\alpha 7$ nAChR (Waldburger et al., 2008). The present study thus extends the current knowledge in showing the expression patterns for mononuclear-like and fibroblast-like cells within the OA synovial tissue and is complementary concerning the delineation of the expression pattern for the synovial lining layer.

The existence of not only ACh production (Grimsholm et al., 2008) but also $\alpha 7$ nAChR in fibroblast-like cells in the OA synovial tissue can be of functional importance. Stimulation of ACh receptors on pulmonary fibroblasts leads to an increase in collagen accumulation (Sekhon et al., 2002). It can also not be excluded that the $\alpha 7$ nAChR may be related to attempts for repair. In studies on skin wound healing, it was thus shown that the $\alpha 7$ nAChR is time-dependently expressed in distinct skin cell types, which may be closely involved in

the repair processes of the skin wound (Fan et al., 2011). The $\alpha 7$ nAChR is also described to contribute to the wound repair of respiratory epithelium (Tournier et al., 2006). Furthermore, an up-regulation of the cholinergic system is reported to be involved in the stimulation of collagen deposition during wound healing (Jacobi et al., 2002). The occurrence of anti-inflammatory effects via cholinergic effects on inflammatory cells is frequently documented (e.g. Kawashima and Fuji., 2008). The *in vitro* studies performed by Waldburger and collaborators showed that both synovial fibroblast-like cells and peripheral macrophages respond to cholinergic stimulation leading to inhibition of pro-inflammatory cytokines (Waldburger et al., 2008).

8. Conclusion

The present study adds new information on the expression patterns of the $\alpha 7$ nAChR for synovial tissue, namely for this tissue in OA. The results presented here, coupled to the finding that there is evidence favouring the occurrence of synthesis of ACh in OA synovial tissue (Grimsholm et al., 2008), imply that the non-neuronal cholinergic system should be further considered for the OA affected joint. It is likely that non-neuronal ACh can have its effects on the $\alpha 7$ nAChR in the OA synovial tissue. Similarly, it is considered that the locally produced ACh in the airways targets ACh receptors located in the airway region where the ACh is produced (Racké et al., 2006).

One possibility is that the production of ACh in non-neuronal cells is related to the occurrence of a great demand on the tissue. That is discussed as one possibility to explain the much more marked ChAT expression in tenocytes of patients with chronic painful tendons than in tendons of normal subjects (Forsgren et al., 2009). Tissue organization and function is hereby influenced. Concerning OA, it would therefore be of interest to know if there is a cholinergic component concerning the cartilage destruction that occurs. It can hereby be noted that less cartilage destruction, as well as on the whole a milder arthritis, was observed for mice lacking the $\alpha 7$ nAChR in studies on collagen-induced arthritis (Westman et al., 2010). It might be that the increased production of ACh in the tissue initially is an attempt to "rescue" the tissue, but that long-standing cholinergic upregulation can contribute to deterioration of the tissue. That was suggested to be the case for the chronic painful tendons (Forsgren et al., 2009). Effects of ACh on fibroblasts and myofibroblasts in chronic obstructive pulmonary disease are considered to be involved in remodelling of the tissue (Haag et al., 2008; Racké et al., 2008).

Interference with the effects of ACh, mainly via influences on the $\alpha 7$ nAChR, may be a new strategy of value in the treatment of arthritis (van Maanen et al. 2009b,c; Westman et al., 2009; Bruchfeld et al., 2010; Pan et al., 2010; Zhang et al., 2010). There are several lines of evidence which suggest that $\alpha 7$ nAChR agonists can inhibit the proinflammatory cascade that occurs in arthritis. Selective $\alpha 7$ nAChR agonist decreases the production of IL-6 by IL-6 stimulated fibroblast-like synoviocytes and the reduction of production from these cells of several cytokines/chemokines via ACh was blocked by a $\alpha 7$ nAChR antagonist (Waldburger et al., 2008). It, however, remains to be clarified as to whether $\alpha 7$ nAChR agonist treatment is valuable in the chronic long-lasting stages of arthritis.

The overwhelming part of the studies favouring a functional importance of the cholinergic system and the possible usefulness of interfering with the $\alpha 7$ nAChR are performed on RA. It should here be recalled that some RA specimens from chronic stages of severe RA

contained an abundance of ChAT immunoreactive cells (fibroblast-like and monuclear-like cells) in our previous study (Grimsholm et al., 2008). The present study reinforces that a non-neuronal cholinergic system, including presence of the $\alpha 7$ nAChR in the synovium, should be further considered also for OA.

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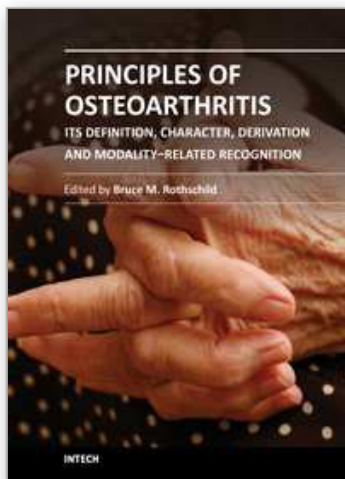
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Principles of Osteoarthritis- Its Definition, Character, Derivation and Modality-Related Recognition

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This volume addresses the nature of the most common form of arthritis in humans. If osteoarthritis is inevitable (only premature death prevents all of us from being afflicted), it seems essential to facilitate its recognition, prevention, options, and indications for treatment. Progress in understanding this disease has occurred with recognition that it is not simply a degenerative joint disease. Causative factors, such as joint malalignment, ligamentous abnormalities, overuse, and biomechanical and metabolic factors have been recognized as amenable to intervention; genetic factors, less so; with metabolic diseases, intermediate. Its diagnosis is based on recognition of overgrowth of bone at joint margins. This contrasts with overgrowth of bone at vertebral margins, which is not a symptomatic phenomenon and has been renamed spondylosis deformans. Osteoarthritis describes an abnormality of joints, but the severity does not necessarily produce pain. The patient and his/her symptoms need to be treated, not the x-ray.

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