CMLS, Cell. Mol. Life Sci. 59 (2002) 5–18
1420-682X/02/010005-14 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Molecular pathology and pathobiology of osteoarthritic cartilage

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Abstract. The biochemical properties of articular cartilage rely on the biochemical composition and integrity of its extracellular matrix. This matrix consists mainly of a collagen network and the proteoglycan-rich ground substance. In osteoarthritis, ongoing cartilage matrix destruction takes place, leading to a progressive loss in joint function. Beside the degradation of molecular matrix components, destabilization of supramolecular structures such as the collagen network and changes in the expression profile of matrix molecules also take place. These processes, as well as the pattern of cellular reaction, explain the pathology of osteoarthritic cartilage degeneration. The loss of histochemical proteoglycan staining reflects the damage at the molecular level, whereas the supramolecular matrix destruction leads to fissuring and finally to the loss of the cartilage. Chondrocytes react by increasing matrix synthesis, proliferating, and changing their cellular phenotype. Gene expression mapping in situ and gene expression profiling allows characterization of the osteoarthritic cellular phenotype, a key determinant for understanding and manipulating the osteoarthritic disease process.

Key words. Cartilage; osteoarthritis; collagen; proteoglycan; apoptosis; mRNA expression; cDNA array.

Introduction

Osteoarthritis is the most common disabling human condition in the western world. It is not a single disease entity, but represents a disease group with rather different underlying pathophysiological mechanisms. In this review, we will only discuss degenerative joint disease of the large weight-bearing joints, focussing on the knee joint, and not entities such as Aberdeen's arthrosis of the fingers, for example. We do not intend to address secondary osteoarthritis due to genetic defects and metabolic disorders or to discuss the development and biology of secondarily formed cartilage within osteoarthritic joints, such as osteophytes and cartilage islands in the eburnated bone plate. Rather, we intend to focus on some aspects of the cellular and biochemical changes which have been established in the last few years and which shed light on the underlying disease processes. In this context, we will discuss our own data and those of others, with particular reference to cell differentiation and matrix anabolism. For detailed discussions of degradative processes, the reader is referred to other recent work [1, 2]. New technologies for exploring novel triggering, mediating, and perpetuating molecular factors involved in joint failure round off our review of the molecular pathology and pathobiology of osteoarthritic cartilage.

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Morphology, cell biology, and biochemistry of normal adult articular cartilage

Articular cartilage is a highly specialized and uniquely designed bio-material that forms the smooth, gliding surface of the diarthrodial joints. It is an avascular, aneural, and alymphatic matrix which is synthesized by the sparsely distributed resident cells – the chondrocytes [3]. At the supramolecular level, cartilage matrix consists of two basic components: a fibrillar and an extrafibrillar matrix. The fibrillar matrix is a network consisting mainly of type II collagen, together with other collagens, predominantly type IX and type XI [4]. Type VI collagen has only been found in the pericellular matrix, directly surrounding the chondrocyte [5–7]. The nonfibrillar component consists predominantly of highly sulfated ag-

grecan monomers, attached to hyaluronic acid and link protein, forming very large, polyanionic aggregates. In terms of the physical properties of cartilage matrix, tensile strength comes from the collagen network, which hinders expansion of the viscoelastic aggrecan component, providing compressive stiffness [8]. Under compressive force, cartilage matrix is compliant, rapidly recovering its elasticity as water is drawn back into the matrix by the hydrophilic aggrecan aggregates [9].

Cartilage matrix also contains a large number of other components that are important for matrix cohesion and for regulation of chondrocyte function. The small nonaggregating proteoglycans decorin, biglycan, and fibromodulin have all been detected in cartilage matrix. Evidence suggests that decorin and fibromodulin may be involved in regulation of fibrillogenesis and also in growth factor binding [10–12]. A role for biglycan has yet to be determined. Other matrix proteins that have been detected in articular cartilage matrix include fibronectin, cartilage matrix protein, and cartilage oligomeric protein, which may be involved in regulation of the cellular gene expression pattern and the chondrocytic phenotype.

The cartilage matrix itself can essentially be divided into four separate zones: the superficial, middle, deep, and calcified zones [13]. In adult articular cartilage, cells of the superficial layer are small and flat and are orientated with the collagen fibers. Chondrocytes of the middle zone appear larger and more round, with an apparently random distribution within the matrix. The direction of the collagen fibers is also more randomly structured within the middle zone. Within the deeper zones, the cells form in columns, lying perpendicular to the cartilage surface, as do the collagen fibers. Chondrocytes themselves appear to behave differently depending on their position with the different layers of the cartilage matrix [14, 15].

Normal turnover of cartilage matrix

In normal human articular cartilage, aggrecan and link protein exist as a heterogeneous population [16–18], differing in size and composition as a result of differential posttranslational glycosylation and proteolysis [19]. Normal proteolytic aggrecan turnover is highly regulated and is most probably implemented by the action of matrix metalloproteinases (MMPs), particularly MMP3 [20]. However, a second cleavage site, the 'aggrecanase' site has also been described in the interglobular domain and contributes to the proteolytic cleavage of aggrecan [21, 22]. In normal adult articular cartilage, the turnover of aggrecan is not excessive and the half-life of aggrecan monomers and aggregates depends very much on the matrix compartment looked at, ranging from days to months [23]. Subdomains of the aggrecan core protein have even been measured to persist for years [24].

The collagen type II network is extremely stable. The tightly wound triple helices that constitute the collagen fibers are further stabilized by a high degree of cross-linking, which steadily increases with age [25]. Fiber destabilization can only be brought about by cleavage of the triple helix due to the action of collagenases, the most likely candidates for type II collagen being MMP-1 and MMP-13 [26, 27].

Finally, changes in the biochemistry of articular cartilage and deterioration in cartilage matrix function result from diverse actions including age, loading, genetics, trauma, and so on. Investigation of the effects of these factors, both in vivo and in vitro, provides important clues as to the processes at work in the onset and progression of degenerative joint disease.

Basic pathology – changes in the extracellular matrix of articular cartilage

Primary osteoarthritis of the large weight-bearing joints is generally a result of imbalance in the physicochemical resisting properties of the articular cartilage and applied mechanical stress. Finally, it consists of the destruction and failure of the extracellular matrix, the functional element of articular cartilage.

Macroscopically, osteoarthritic cartilage is often yellowish or brownish and is typically soft. The surface shows roughening in the early stages and overt fibrillation and matrix loss in the later stages until the eburnated subchondral bone plate is visible. Beside the degradation of molecular components, destabilization of supramolecular structures also takes place. For example, destabilization of the collagen network results in microscopically and finally macroscopically visible matrix destruction. Both mechanical wear and enzymatic degradation appear to play a pivotal role during the disease process. Additionally, missing or discoordinate matrix anabolism as well as alterations in the matrix gene expression profile of the chondrocytes are important issues in the disease process. Together, this leads to osteoarthritic cartilage matrix destruction at the molecular (e.g., proteoglycan depletion), the macromolecular (e.g., network loosening), the microscopic (e.g., fissuring), and the macroscopic (e.g., cartilage tear) level. Thus, the molecular degradation mechanisms and the cellular reaction pattern discussed below explain the pathomorphology of osteoarthritis.

Biochemistry of the interterritorial matrix

The destruction of articular cartilage and the loss of its biomechanical function are largely equivalent to the destruction and loss of the interterritorial cartilage matrix. This mainly implicates the catabolic imbalance between

degradation and resynthesis of matrix components at the molecular and supramolecular level despite the attempt of the chondrocytes to compensate for it. So far, our knowlegde focusses on changes in the two major components of the interterritorial cartilage matrix, the collagen network and the interwoven proteoglycan aggregates. Loss of aggrecan or, more exactly, of fixed charges is a feature in early stages of cartilage degeneration, whereas the overall content of collagen remains rather constant throughout the disease process [28, 29]. We still do not know what comes first: a loss of proteoglycans or a loosening of the collagen network, as both finally implicate the other as well. As shown by Maroudas [8], a loosening of the collagen network leads to a loss of proteoglycans, and a loss of proteoglycans leads to mechanical overload and thus damage and loosening of the collagen network. In particular, the latter appears to be responsible for the hyperhydration of articular cartilage in the early phases of the disease process, macroscopically visible as softening and swelling of the osteoarthritic articular cartilage [8]. Degradation processes appear to be specifically prominent in the surface zone and around the chondrocytes in osteoarthritic cartilage [30, 31]. Enhanced levels of many MMPs including MMP-7 [32], membrane-type I MMP [33], MMP-8 [34, 35], 'aggrecanase' [36], ADAM-10 [37], and ADAM-15 [38] have been reported to accompany the increased matrix degradation in osteoarthritic cartilage.

Beside changes in the quantitative amounts and in the degradation of cartilage components, alterations of cartilage components or the expression of molecules which are not present in normal articular cartilage also appear to be important phenomena. Thus, the composition of the proteoglycans has been shown to change in osteoarthritic cartilage, such as an increase in the size of the proteoglycan molecules with a reduction in keratan sulfate relative to chondroitin sulfate side chains. We and others reported the appearance of molecules in osteoarthritic cartilage such as tenascin [39] and, collagen types IIA [40] and III (fig. 1C, F) [41], which are barely seen or are undetectable in normal articular cartilage. Whether the newly synthesized type IIA and III procollagens have any special functional role in osteoarthritic cartilage or represent functionless matrix deposits remains a matter of specula-

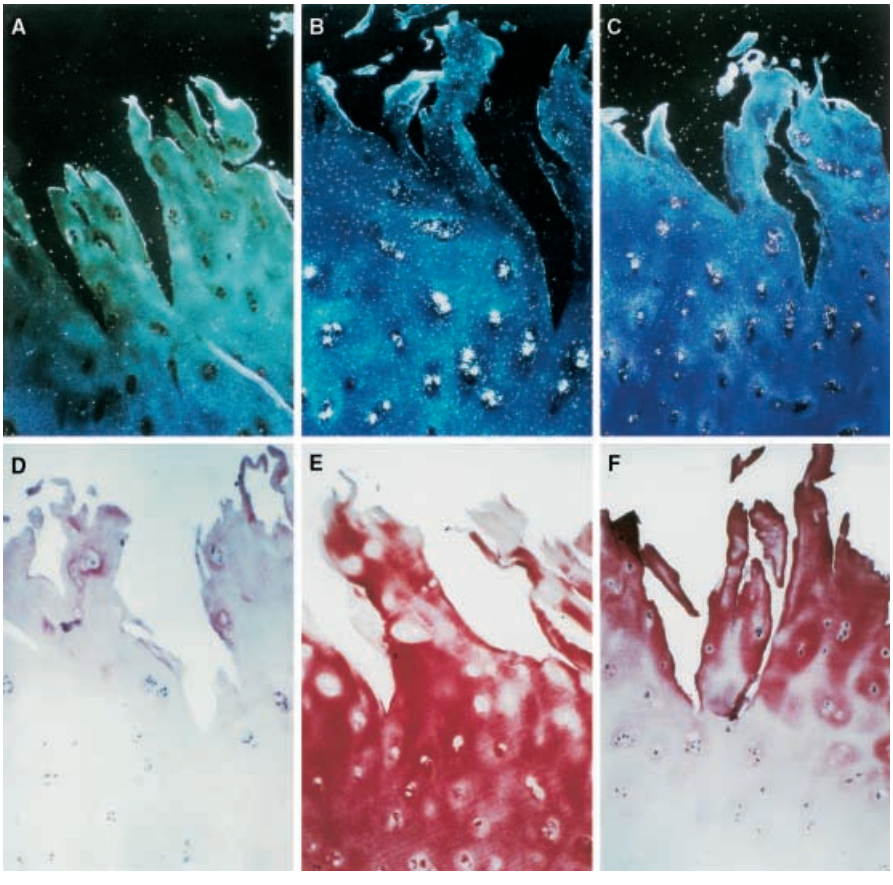

Figure 1. In situ hybridization analysis of collagen expression (*A–C*) and (immuno)histochemical staining of collagens (*D–F*) in sections of human osteoarthritic cartilage. For detection of cytoplasmic mRNA, cartilage sections were hybridized with ^{35}S-RNA probes specific for α1(I) (*A*), α1(II) (*B*), or α1(III) (*C*). Matrix collagens were immunostained with anti-type I (*D*), anti-type II (*E*) and anti-type III (*F*) antibodies, followed by alkaine phosphatase-labeled secondary antibodies.

tion at the moment. Sooner or later they would become degraded and would diffuse out into the cartilage matrix and finally the synovial fluid. Type X collagen becomes a prominent component in the very deep and calcified cartilage zones [42]. There, it might be directly involved in the ongoing calcification process in these zones, which is characteristic for osteoarthritic cartilage.

We recently showed that type VI collagen, which in normal articular cartilage is largely restricted to the pericellular matrix component, is also strongly increased in the interterritorial matrix of osteoarthritic articular cartilage. However, whether this indicates the presence of a type VI collagen network in this matrix compartment [43] remained unclear. Alternatively, the epitope positivity could derive from diffusion of type VI collagen degradation products [44, 45].

Pathobiochemistry of the pericellular matrix

Most investigations have so far analyzed the (patho)biochemistry of the interterritorial cartilage matrix. Much less attention has been paid to changes in the pericellular cartilage matrix. At present, only the changes to type VI collagen have been investigated thoroughly.

Type VI collagen is concentrated in the pericellular matrix in normal and diseased cartilage [5, 6, 46]. Our own ultrastructural studies have shown a physical overlap of the type VI collagen network with the type II collagen-positive matrix, suggesting that type VI collagen is one central molecular component forming a mechanical interface between the rigid type II matrix and the cells. Poole and others [7, 31, 47, 48] have shown that the type VI-positive pericellular matrix is severely altered in osteoarthritic cartilage. Both increased synthesis as well as enhanced degradation of collagen type VI are well documented in osteoarthritic cartilage [44, 45]. In fact, type VI collagen-degrading enzymes, either synthesized by the chondrocytes themselves or diffusing in from the synovial space, might explain the reduced type VI collagen staining observed in the superficial zone of osteoarthritic cartilage [7]. Nonhomogeneous degradation of type VI collagen is most likely the reason for the nonhomogenous distribution of different type VI collagen chains observed in double-labeling experiments in osteoarthritic, but not normal articular cartilage [7]. Ultrastructural analysis showed abundant cross-striated fibrous type VI collagen aggregates, in particular around osteoarthritic chondrocytes [6, 44, 49, 50] which also most likely reflects partial degradation of type VI collagen. Such a transformational conformation of resident type VI collagen rather than some sort of net loss of collagen type VI molecules might be the primary reason for a significant functional derangement of the pericellular cartilage matrix in osteoarthritic cartilage.

The consequences of the derangement of the pericellular matrix in osteoarthritic cartilage are unknown. It might impede proper cell-matrix interaction, e.g., via integrins [51–53], and the cell might loose its protective basket against compression forces. Alterations in the type VI collagen microenvironment can also influence the synthetic activity of chondrocytes and, thus, modulate e.g., proteoglycan synthesis. Finally, alterations in the pericellular microenvironment can play a role in chondrocyte proliferation leading to chondrocyte clustering typical for osteoarthritic cartilage [29, 47, 54].

Basic pathology – cellular reactions

The cellular reaction pattern during the osteoarthritic disease process is at first sight rather pleomorphic. However, it can be basically summarized in three categories. First, the chondrocytes can undergo cell death, whether programmed (apoptosis) or not (necrosis), or they can proliferate to compensate for cell loss or to increase their synthetic activity, as cells do in many other tissues of the body. Second, chondrocytes activate or deactivate their synthetic-anabolic activity by increasing or decreasing anabolic gene expression. Finally chondrocytes undergo phenotypic modulation implicating an overall severely altered gene expression profile of the cells in the diseased tissue.

Proliferation – necrosis – apoptosis

Lacuna emptying and cell cloning are typical histological features of osteoarthritic cartilage [55, 56]. Several studies [28, 57, 58] have clearly shown that there is (very low) proliferative activity in osteoarthritic chondrocytes in contrast to normal articular chondrocytes, which do not show any proliferative activity, explaining chondrocyte cloning observed in osteoarthritic cartilage. The increased proliferative activity of chondrocytes, which is found primarily in the upper cartilage zones, might well be due to better access of the chondrocytes in these areas to proliferative factors from the synovial fluid due to fissuring or loosening of the collagen network [59]. Alternatively, damage to the collagen matrix integrity, which is particularly impaired in the upper zones of osteoarthritic cartilage [60], might contribute to the proliferative activity of osteoarthritic chondrocytes. Chondrocyte proliferation resulting in a higher cellular content could represent a cellular reaction to cartilage destruction, but this is unlikely to represent efficient tissue repair [58], as the clusters in the very upper zone do not appear to add significantly to matrix anabolism [61].

Based on the observation of significant numbers of empty lacunae at the light microscope level and cellular

debris-like material at the ultrastructural level in osteoarthritic cartilage, some authors have suggested that cell death is a central feature in osteoarthritic cartilage degeneration [55, 62]. Recently, apoptotic cell death was reported to be a dominant event in osteoarthritic cartilage degeneration, although in a contradictory manner [63–66]. Major cell death would easily lead to a failure in cartilage matrix turnover, because chondrocytes are the only source of matrix component synthesis in articular cartilage. In our own studies, we confirmed that apoptosis occurs in osteoarthritic cartilage, but at a very low rate. Neither in aged nor in lower-grade osteoarthritic human adult articular cartilage is a significant amount of empty cell-free lacunae in the noncalcified cartilage detectable using confocal microscopy of thick cartilage slices. This suggests that chondronecrosis has only a limited impact on the pathology of early osteoarthritis or aging of human articular cartilage [28].

The only zone in which a large number of empty lacunae was found was the calcified cartilage layer. The continuous progression of cartilage calcification in osteoarthritic cartilage might explain, at least in part, the increasing number of empty lacunae reported, particularly in high-grade lesions [56, 62]. The highly reduced number of living chondrocytes in the calcified layer seems not to impair articular cartilage function under normal conditions, but might well play a detrimental role in osteoarthritis particularly of more advanced stages. Thus, it might be responsible for the horizontal cracking of cartilage matrix just above this zone, which is sometimes seen in osteoarthritic cartilage [55].

Metabolic activation and hypoanabolism

In osteoarthritic cartilage, a number of biochemical studies have demonstrated enhanced synthesis of extracellular matrix components (figs. 1B, C, 2B–D) [67–73]. Chondrocytes attempt to repair the damaged matrix by increasing their anabolic activity. The fact that type II collagen expression appears to be much more upregulated than aggrecan, for example [68, 73–76] mimics fetal [77] and osteophytic cartilage [78]. In both of these tissues, chondrocytes have to synthesize and assemble a new extracellular matrix consisting largely of collagen type II. Both are in contrast to normal articular cartilage in which chondrocytes only need to control tissue homeostasis by maintaining a stable matrix composition. This mainly involves the control of proteoglycan turnover, whereas collagen type II turnover is presumably nearly zero [79].

Despite the increased biosynthetic activity of chondrocytes, a net loss of proteoglycan content is one of the hallmarks of all stages of osteoarthritic cartilage degeneration [28]. This leads to the assumption that overall enzymatic degradation of matrix components might be the reason for the metabolic imbalance in osteoarthritic cartilage [80]. However, most previous studies were based on an overall measurement of chondrocyte behavior or matrix composition within the entire osteoarthritic cartilage. The applied techniques did not allow the detection of differences between cells of different cartilage zones. In situ analyses showed that the loss of fixed charges occurs exactly in the upper zones of osteoarthritic cartilage, in which the cells downregulate their expression of matrix components including aggrecan, whereas the cells of the deeper zones are still activated (fig. 2B–D) [61, 74,

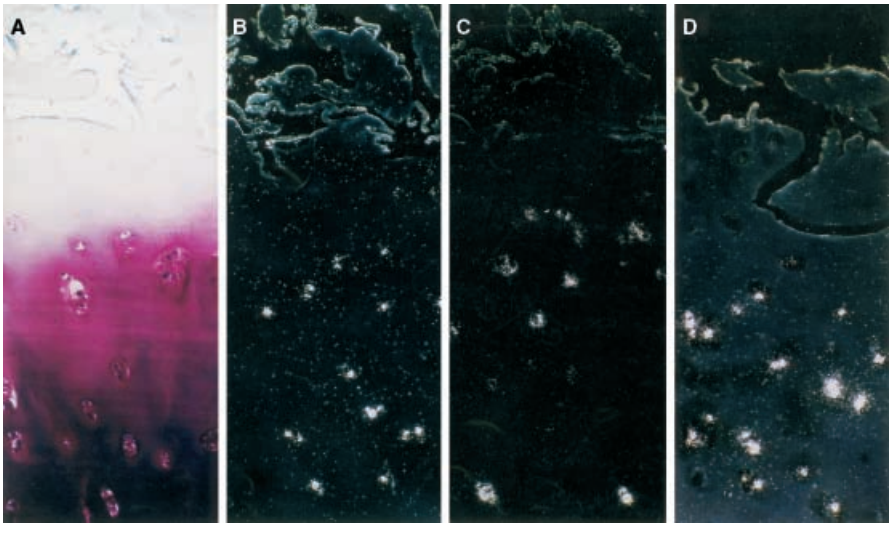

Figure 2. Enhanced expression of aggrecan core (*B*) and link protein (*C*) as well as collagen type II (*D*) mRNA in chondrocytes of the middle zone of moderately damaged cartilage (Mankin's grade 5). The upper-zone chondrocytes did not show expression of these matrix components. Corresponding to the suppression of aggrecan mRNA expression, depletion of proteoglycans of the extracellular matrix in these upper areas was observed (*A*).

81]. This explains, at least in part, the loss in proteoglycan content in this zone, if one assumes that the diffusion capacity of aggrecan monomers is limited and enhanced synthesis in one zone cannot compensate for the synthetic failure in adjacent zones.
Notably, even in specimens with a very high Mankin grade (>8) implicating an advanced disease state, some chondrocytes expressing aggrecan, link protein, and collagen type II mRNA were observed [61]. Thus, even in advanced stages of cartilage degeneration, chondrocytes maintain their capacity for anabolic activity.

Phenotypic alterations

Phenotypic changes are a central feature of chondrocytes. This is known from many studies of chondrocyte differentiation in vivo in the fetal growth plate cartilage, but even more so from analyses of chondrocyte behavior in vitro [for a review see ref. 82]. Thus, the chondrocyte phenotype is not stable in vitro, in particular in monolayer culture. Several factors such as retinoic acid, bromodeoxyuridine, or interleukin (IL)-1 induce so-called 'dedifferentiation' or modulation of chondrocytes to fibroblast-like cells. They stop expressing aggrecan and collagen type II though they are still very active cells and express collagen types I, III, and V, for example [83–85]. This example clearly demonstrates the implications of phenotypic alterations of chondrocytes: despite potentially high synthetic activity, 'dedifferentiated' chondrocytes do not express cartilage-specific genes such as aggrecan or type II collagen. Therefore, beside deactivation, phenotypic alterations represent the second potential reason for the anabolic failure of chondrocytes in osteoarthritic cartilage.

The osteoarthritic chondrocyte – an osteoarthritic phenotype?

As mentioned above, several different phenotypes of chondrocytes are known to occur in vitro, during fetal development in vivo, and potentially also in the disease process. Classically, these phenotypes are categorized largely by subtyping of collagen gene expression [82, 86]: Thus, *chondroprogenitor* cells are characterized by the expression of the alternative splice variant of type II collagen COL2A [77, 87]. *Mature* chondrocytes express the typical cartilage collagen types II (COL2B), IX, and XI as well as aggrecan and link protein [77, 88, 89]. *Hypertrophic* chondrocytes are marked by the expression of type X collagen. These cells are physiologically found in the lowest zone of the fetal growth plate cartilage [90, 91] and in the calcified zone of adult cartilage, which is thought to be a remnant of the lower hypertrophic zone of the fetal growth plate cartilage [92]. Chick chondrocytes have been shown to be able to undergo *post-hypertrophic* differentiation to osteoblast-like cells, which specifically express type I collagen [93–95]. Another phenotype, which has so far only been described in vitro [84, 85, 96] and in chondrogenic neoplasms [97], is the so-called *dedifferentiated* chondrocyte. This cell is of typical spindle-like or stellate shape and synthesizes collagen types I and III, but not the cartilage-typical collagen subtypes or aggrecan [83–85].
Below, we will review results of the phenotypic analysis of osteoarthritic chondrocytes based on collagen expression studies mostly by in situ detection methods at the mRNA (in situ hybridization) and protein (immunohistochemistry) levels. However, it will be important to characterize further the 'osteoarthritic' cellular phenotype on a broader molecular basis, which will also provide additional markers to monitor cellular disease processes. Three promising approaches will be outlined which we have used in our laboratory to identify genes involved in the disease process: (i) cDNA array technology, which allows the screening of many (known) genes at the same time (fig. 3), (ii) determination of gene expression levels in minute amounts of tissue by quantitative PCR (fig. 4), and (iii) differential display technology, which enables characterization of new genes involved in the disease process.

In situ expression analysis

In our laboratory, we performed in situ expression analyses in normal and osteoarthritic cartilage specimens using the markers for chondrocyte differentiation mentioned above: collagen type II and aggrecan (activated functional chondrocytes), collagen types I and III (dedifferentiated chondrocytes), collagen type IIA (chondroprogenitor cells), and collagen type X (hypertrophic chondrocytes). These analyses showed activated chondrocytes mostly in the middle zones of osteoarthritic cartilage [61, 74]. Type III collagen-expressing cells were mainly found in the upper middle zone cells, but not in the deeper portions of the middle and deep zone (fig. 1C, F) [41]. Type IIA procollagen was also expressed and synthesized by some of the osteoarthritic chondrocytes and was deposited primarily in the cell-associated cartilage [40], indicating at the molecular level that adult articular chondrocytes start to reexpress a fetal chondrocytic phenotype [87, 98]. A reversion to a fetal phenotype and the reinitiation of the fetal skeletal developmental processes also occurs in the deepest zones of osteoarthritic cartilage; here, the cells start to express type X collagen [43] and undergo (apoptotic) cell death (see above) and the cartilage matrix calcifies. All these events mimic processes taking place in the lowest zone of fetal

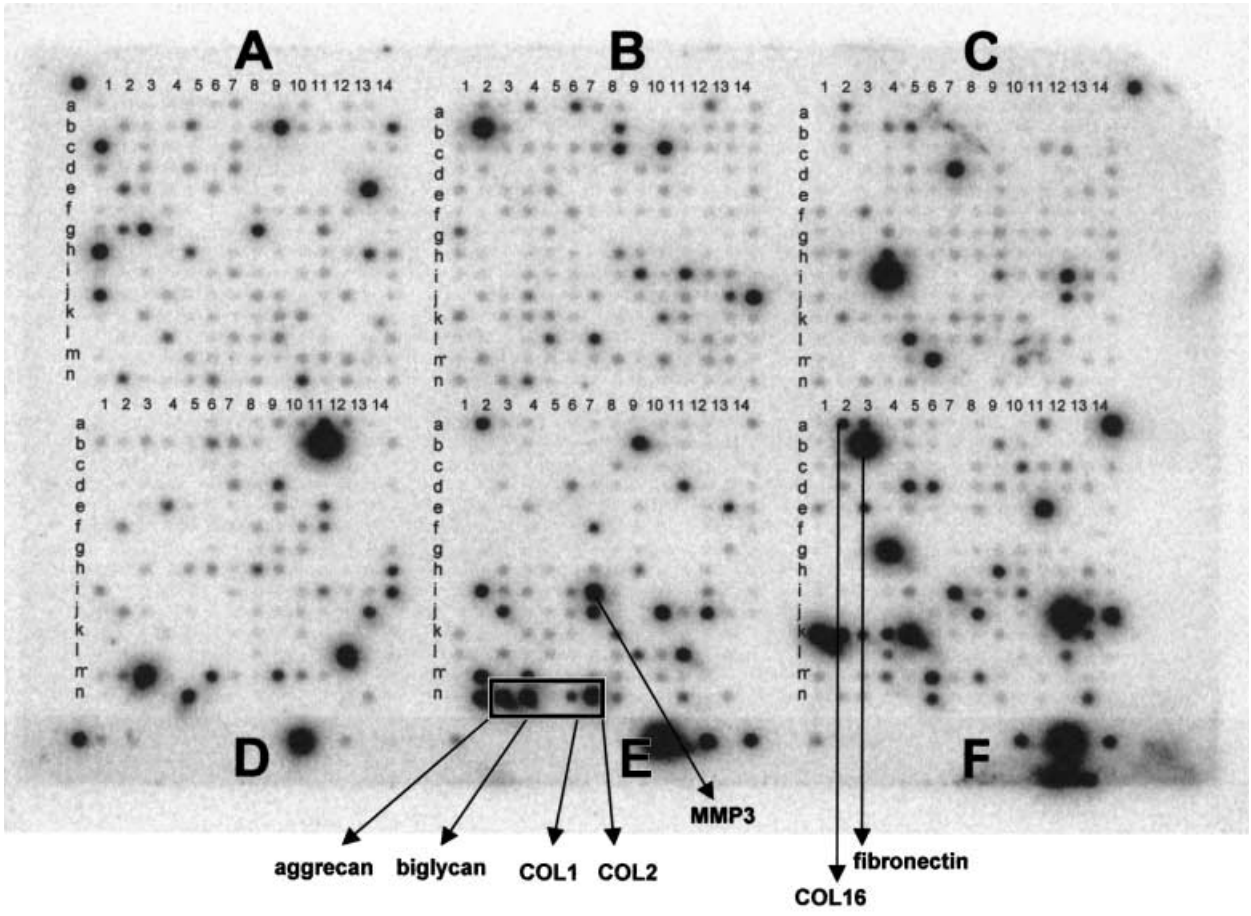

Figure 3. RNA (5 µg) isolated from normal human articular cartilage obtained from a 72-year-old female donor was hybridized with a Human Cancer 1.2 Atlas cDNA array (Clontech). Arrayed quadrants (A–F) contain spotted cDNA for specific gene groups. (*A)* Tumor suppressors, transcription factors and related oncogenes, serine/tyrosine kinases, non-receptor tyrosine kinases, intracellular signal transduction-related oncogenes, cyclins, and cell cycle regulators. (*B)* Receptor-associated proteins, intracellular kinase network proteins, tyrosine phophatases, and signal transduction modulators. (*C)* Caspases, Bcl family proteins, apoptosis proteins, and transcription factors. (*D)* Cell surface antigens, adhesion molecules, growth factor, chemokine and cytokine receptors, and stress response proteins. (*E)* Growth factors and cytokines, protein turnover (MMPs). (*F)* Immune system proteins, metabolic pathways, cytoskeleton, and motility. (*E, F*) Signals for aggrecan, biglycan, collagen I, II, and XVI , fibronectin and MMP-3 are indicated.

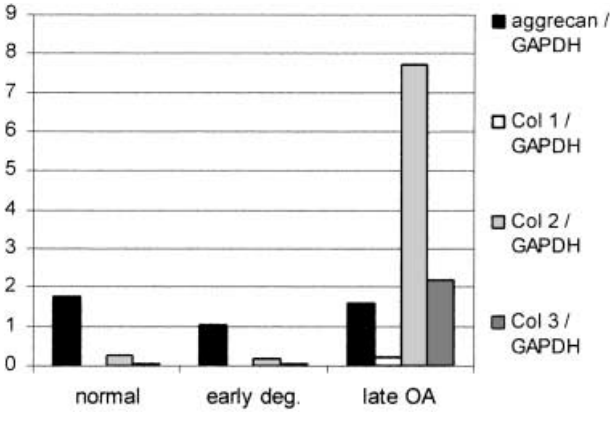

Figure 4. Quantitative PCR analysis showing measurement of aggrecan and collagen types I, II, and III in relative ratios to GAPDH in normal, early degenerative, and late-stage osteoarthritic cartilage.

growth plate cartilage. Notably, no expression of type I collagen mRNA was found in any zone of normal or osteoarthritic articular cartilage (fig. 1A, D) [41, 80].

The uppermost chondrocytes often showed no expression of any collagen type investigated (figs. 1, 2). This is in contrast with the established modulations of the chondrocyte phenotype known in vivo and in vitro, all of which express at least one of the discussed collagen types. In fact, we are largely lacking specific markers for these cells, although one good candidate could be the superficial zone protein [99]. This further stresses the need to establish a broader gene expression profile by modern screening technologies.

Gene expression profiling – cDNA array technology – quantitative PCR

The cellular reaction pattern underlying the changes in the metabolic activity of the chondrocytes in osteoarthritic cartilage degeneration is poorly understood. One reason is that many of the genes involved are not yet identified and characterized. Gene expression profiling using cDNA arrays is a powerful emerging technology for identifying differences in mRNA expression levels of large numbers of genes at the same time [100]. The major limitation in using this technology, however, is the inherent heterogeneity of cell populations present in most tissues. Thus, one cannot distinguish expression signals derived from all sorts of other cells physiologically or pathologically present in the tissues and contributing in unclear ratios to detected signal levels. In this respect, adult human articular cartilage offers the great advantage of comprising only one cell population. Thus, detected gene expression levels can be fully attributed to the chondrocytes.

In our laboratory, we first established a homemade array with little more than 20 cartilage-relevant genes for testing the method. Subsequently, we used the human cancer 1.2 array from Clontech, which offers the possibility to screen 1176 genes simultaneously. The majority of genes never revealed a positive signal (e.g., epithelia- or lymphocyte-specific genes) and could serve as excellent internal negative controls. In contrast, many genes, which are known to be expressed by most cells (e.g., housekeeping genes) or by chondrocytes (aggrecan, type II collagen), could serve as internal positive controls. However, some genes, which are known to be expressed in articular chondrocytes, did not reveal positive signals, stressing limitations in the detection sensitivity of the applied technique. A very powerful technique to confirm the array results even with the minor amounts of RNA available in the case of RNA directly isolated form articular cartilage was the introduction of the TAQMAN technology (Perkin-Elmer), which uses an internal, target-specific fluorochrom-labeled probe to quanitfy online mRNA amplification products (fig. 4). This technique allowed us to quantify mRNA expression levels from RNA amounts isolated from less than 20 mg of human adult (normal or osteoarthritic) articular cartilage [101].

Our cDNA array experiments confirmed on a broad level previously published data on many characteristic features of osteoarthritic chondrocytes, but also showed new features not previously described [Aigner et al., in press]. Thus, type XVI collagen appears to be a new member of the collagenous cartilage matrix. A molecule known to be reexpressed in osteoarthritic cartilage while being absent in normal adult articular cartilage [39], is tenascin. A very prominent gene expression product of normal chondrocytes was fibronectin. Fibronectin was increased in osteoarthritic chondrocytes. This is particularly interesting, because fibronectin fragments are suspected to be catabolic regulators of cartilage matrix turnover [102]. Matrix-degrading proteases were also found to be strongly regulated, e.g., MMP-3, which appeared to be upregulated early in the degeneration process. In late stage osteoarthritic cartilage, a significant increase in MMP-13 and MMP-2 expression was found, suggesting these enzymes are involved in the breakdown of collagen fibers in the late stages.

Despite considerable limitations, such as limited sensitivity and insensitivity to alternative splicing, posttranscriptional regulation, and translational modification, the cDNA array technology provides a powerful tool to obtain an overview of the gene expression pattern, virtually unachievable with other techniques.

Gene expression profiling in vitro

Under normal conditions, adult articular chondrocytes are generally quiescent and hence gene expression levels associated with normal catabolic and anabolic processes would be extremely difficult to profile. However, in the presence of cytokines and growth factors, gene expression can be effectively hyperstimulated, providing in vitro models which are ideally suited to gene expression technologies such as profiling using cDNA arrays. We have carried out in vitro gene expression profiling studies using total RNA isolated from human articular chondrocytes stimulated with the catabolic cytokines IL-1β and tumor necrosis factor (TNF)-α or with serum.

Of the 1176 genes on the cDNA array, over 400 were expressed by chondocytes in vitro, enabling us to obtain a simultaneous overview of both the catabolic and anabolic responses [L. McKenna and T. Aigner, unpublished data]. The picture we have obtained for the catabolic response of the chondrocyte is highly complex and supports a wealth of published data. For example, expression of the key matrix proteins aggrecan and COL2A1 were, as anticipated, reduced, whereas in our anabolic model (effects of serum on chondrocyte metabolism), both were increased. The decrease in matrix protein expression in the cytokine-stimulated cultures was accompanied by expression of mRNA for matrix-degrading proteases such as collagenase-1 (MMP-1) and stromelysin (MMP-3). These enzymes are known to play an important role in destabilizing the cartilage matrix due to proteolytic attack on aggrecan and collagen II molecules [103–106]. The pattern of expression obtained for arrayed transcription factors supported evidence that the catabolic effects of IL-1β and TNF-α on matrix proteins and MMP expression are facilitated via diverse, complex c-jun protooncogene/AP-1 signal transduction pathways [107–109]. For example, mRNA for the c-jun protooncogene was significantly upregulated for cytokine-stimulated sam-

ples together with ERK-1, ERK-2, c-fos and the vav oncogene, another activator of the AP-1 pathway [110]. This selection from our data demonstrates the useful application of array technology to investigating the action of a particular agent on the chondrocyte phenotype. Further analysis of this type of data will help us to gain more of an overview of complex gene expression networks.

How to find new genes – differential display

It is not only expression profiling of known genes that may give us more insights into the complex regulation of cartilage matrix turnover in health and disease. Technologies that identify novel genes are vital to provide us with new targets important in regulation of the chondrocytic phenotype. However, a number of these are not optimal for studies involving human tissue. Differential screening [111–115] and subtractive hybridization [116], require large amounts of pure RNA and extensive libraries, and screening is unidirectional. Poly(A)-PCR subtractive hybridization [116, 117] would enable the isolation of full-length clones and requires smaller amounts of RNA, but problems are frequently encountered when RNA samples demonstrate many similarities, a good library is prerequisite, and screening is also unidirectional. In our laboratory, we chose to use differential display RT-PCR (DD RT-PCR) [118]. The exquisite sensitivity of the PCR reaction made DD RT-PCR a good method to use in the study of gene expression in human articular cartilage and cultured human primary articular chondrocytes, for which isolation of pure RNA in large amounts is barely feasible [101, 119]. Furthermore, differentially expressed genes that are both up- and/or downregulated are displayed in parallel.

DD RT-PCR has been previously used for a number of cell lines and nonhuman systems, including chondrocyte cell lines [120] and embryonic chick sternal chondroctyes [121], and resulted e.g., in the identification of a novel cartilage specific protein, CD-RAP, that is regulated by retinoic acid in bovine articular cartilage [122].

In our laboratory, work is focussed mainly on the phenotypic modulation of human adult articular chondrocytes and the relationship of these changes in gene expression to degenerative joint disease. Therefore, we favored a cytokine- and serum-stimulated model system to maximize the differentially expressed gene pool.

Using this approach, we could identify a number of potentially differentially expressed PCR products [L. McKenna and T. Aigner, unpublished data]. Genes with a known expression profile in articular cartilage included MMP-3, regulated by both IL-1β and TNF-α, which was also in line with our cDNA array hybridization data and also detected in a similar screen by Magerie and coworkers [123]. Previously unidentified cytokine-regulated genes included peptidylglycine α-amidating monooxygenase (PAM) and the enzymes nicotinamide N-methyltransferase and ornithine decarboxylase antizyme.

Identification of potential therapeutic targets is plausible using this approach. For example, PAM, detected in cytokine-stimulated cultures, has not been previously reported in articular cartilage, but in human tracheal chondrocytes [124]. It is the rate-limiting enzyme in the formation of amidated neuropeptides, such as substance-P, which are strongly implicated in the inflammatory process [125], upregulates expression of MMPs, and could contribute to cartilage degeneration in osteoarthritis, all of which make it an interesting candidate for further investigation.

Novel genes have been identified in these screens. Several demonstrate sequence homology with IMAGE clones, although these are not currently functionally annotated. One gene IS-1 (accession AF156968) is upregulated in osteoarthritic chondrocytes, although its potential function remains unclear. With sequencing of the human genome nearing completion, the potential for identification and functional annotation of these novel genes will be dramatically increased.

A basic concept of osteoarthritic cartilage degeneration – future directions

The unique biomechanical properties of articular cartilage are provided by its extracellular matrix and the failure of osteoarthritic cartilage is a consequence of the progressive destruction of this matrix. Overall, (apoptotic) cell death is not a widespread phenomenon in cartilage aging or osteoarthritic cartilage degeneration and values given in the previous literature are significantly overestimated. Thus, is the central problem, at least in the earlier phases of osteoarthritic cartilage degeneration, not (apoptotic) cell death and subsequent cell loss but the metabolic failure of the living cells within the tissue.

In normal adult articular cartilage, the turnover of collagen fibrils is very low, whereas a relatively high turnover rate for aggrecan has been measured. In situ analysis at the single-cell level provides strong evidence that although at first sight osteoarthritic cartilage degeneration appears to be a confusingly heterogeneous process, general rules can be established. Together with other previously published data, our own studies suggest a three-step evolution of cellular events as one central feature during the osteoarthritic cartilage destruction process (fig. 5): (i) an increase in collagen type II and aggrecan synthesis; (ii) modulation of the chondrocytic phenotype with the expression of atypical gene products such as collagen type III; (iii) suppression of aggrecan core protein and collagen type II (and III) mRNA expression with subsequent quantitative loss of aggrecan molecules from the

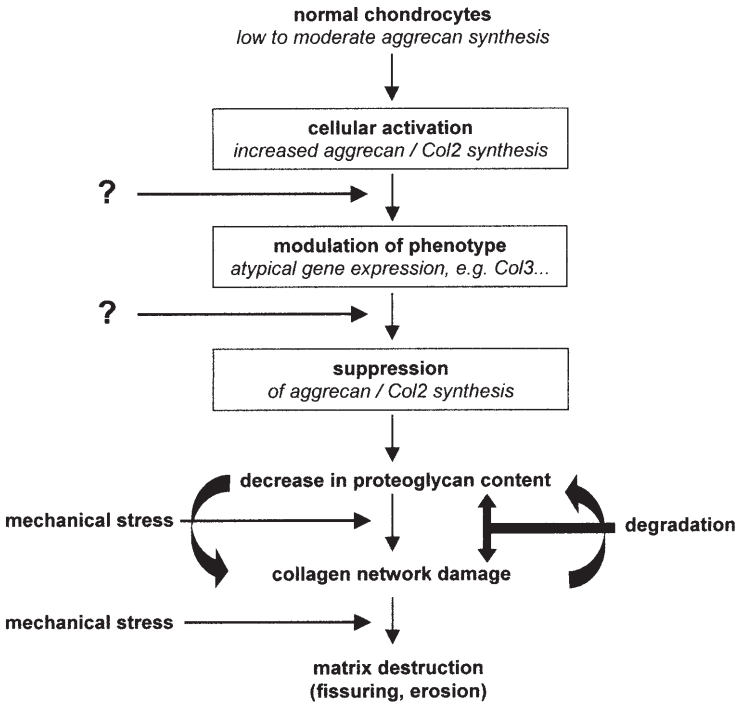

Figure 5. Schematic representation of three steps in a hypothetical pathomechanism involved in the progression of osteoarthritic cartilage degeneration: (i) cellular activation of chondrocytes, (ii) modulation of the cellular phenotype, and (iii) suppression of anabolic activity. The latter leads to a quantitative loss of aggrecan molecules from the extracellular matrix and – via mechanical stress – to collagen network damage, which again promotes further loss of proteoglycans. Finally, fissuring and complete destruction of the cartilage matrix occurs.

extracellular matrix. This results in an increase in the stress applied to the collagen network [8], thus promoting its destruction and thereby further loss of proteoglycans [8]. Physical damage to the collagen network leads to fissuring and complete destruction of the cartilage matrix and cells. This process may begin in the superficial zone and progress to the middle and deeper zones.

This concept does not preclude the importance of proteolytic degradation as a pathogenic mechanism within the osteoarthritic cartilage degeneration process, but rather serves to focus this on the damage to the collagen network [31]. Mature collagen fibers are large, multiply cross-linked rigid structures, which are presumably difficult to repair or replace. Thus, preventing damage to preexisting fibers is a target of primary importance. In contrast, aggrecan, with a faster turnover in normal articular cartilage [126, 127], might more easily be replaced by newly synthesized molecules.

Investigation of early stage disease

Studying the early events during the disease process is of major interest for understanding the mechanisms underlying osteoarthritic cartilage degeneration. The investigation of early stages in humans is for material reasons rather difficult. Most studies – including ours – have been done on peripheral areas of eroded tissue of late-stage osteoarthritic material obtained at surgery. This always assumes that low to moderate Mankin grades [28] of late stages of osteoarthritic joints are comparable to cartilage of central areas of early or moderately advanced osteoarthritis. This, however, is not proven and needs further investigation. In fact, in light of our cDNA array analyses it is rather unlikely.

The osteoarthritic chondrocytic phenotype

Despite some data about changes in the cellular phenotype during osteoarthritic cartilage degeneration, our overall knowledge about these events is still very limited and additional phenotypic markers are mandatory. This will also allow us to understand the underlying regulatory processes and will identify new cellular or intercellular players in the disease process. Very little is known about the latter. And while the dynamic events discussed in this review may partly explain the osteoarthritic process, the factors that initiate it are not clear. These might be of a mechanical or an inflammatory nature leading to limited damage, e.g., to the collagen network. Most probably, several diverse initial events may lead to the same or a similar disease process, and the influence of cytokines such as IL-1 or TNF-α may accelerate this. These mediators can act at a para-/autocrine level or diffuse into the articular cartilage from the synovial fluid.

In vitro systems

A major goal is establishing in vitro systems reflecting the in vivo differentiation patterns of osteoarthritic chondrocytes in order to further analyze the disease process and to develop experimental systems to delay, stop, or even reverse it. This remains a possibility, because chondrocytes, even in severely damaged areas, maintain the capacity to synthesize the necessary cartilage matrix components [61, 70, 71, 75, 128]. Reversibility of switches in activity and differentiation have been demonstrated in vitro as well as in vivo in chondrocytes [129–131]. Therefore, one can assume that if substantial collagen fibril destruction can be prevented, cartilage could repair itself or maintain the status quo due to the potentially high anabolic capacity of its cells [70, 75].

However, one has to keep in mind that reactivation of phenotypically modulated chondrocytes is of little help as it may only enhance noncartilaginous matrix gene expression at the expense of essential matrix components such as aggrecan [132, 133]. This situation would be similar to the enhancement of noncartilaginous protein synthesis of dedifferentiated chondrocytes stimulated by fetal calf serum [134]. Instead, stabilization of the chondrocytic phenotype and redifferentiation of the osteoarthritic chondrocytes is needed to ensure correct matrix anabolism. Factors of the transforming growth factor-β superfamily, in particular bone morphogenetic proteins such as osteogenin [135], might be suitable agents to initiate and promote this.

Acknowledgements. This work was supported by the IZKF (grant D4). We thank Angelika Gehrsitz, Michaela Schäfer, Freya Boggasch, and Pia Gebhard for excellent team work.

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