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voor mijn ouders die mij onbeperkte mogelijkheden bieden

voor Laeta en Winchester die zorgen voor een welkome afleiding

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Chapter 1

General Introduction

General introduction

1 The healthy joint

In diarthroidal joints, two bones meet to allow movement. The normal joint consists of cartilage covering the ends of the bone, to absorb pressure upon load bearing, and to facilitate joint movement by providing a smooth surface. Tendons and ligaments are present to connect the joint. These ligaments are dense bands which extend from bone to bone providing structural support to the joint and limiting the relative motion of adjacent bones (1). The tendons and ligaments are lined with synovial tissue, which produces an ultrafiltrate of the blood: the synovial fluid. This synovial fluid also contains proteins and sugars, such as hyaluronic acid and lubricin, secreted by fibroblast-like synoviocytes that are present in the synovial tissue. The synovial fluid is the main source of nutrients for the chondrocytes of the cartilage (1, 2).

1.1 Articular cartilage

Articular cartilage is a highly specialized, avascular, connective tissue which is located at the end of long bones in diarthrodial-synovial joints. The form and mechanical properties of cartilage are derived from its extracellular matrix. This extracellular matrix is produced and maintained by chondrocytes (the sole cells in cartilage), which occupy only 2-10% of the tissue volume. Collagen type II is the major constituent of the extracellular matrix (10-40% of wet weight) and provides an insoluble fibre network in which other constituents, such as proteoglycans (5-10 % of wet weight), non-collagenous and non-proteoglycan molecules, and chondrocytes, are embedded and imparts tensile strength to the cartilage (*figure 1*) (3-5). There are at least four types of collagens present in the articular cartilage. The predominant type of collagen is type II collagen (90% of total collagen). Other collagens present in cartilage are type IX, type XI and type VI.

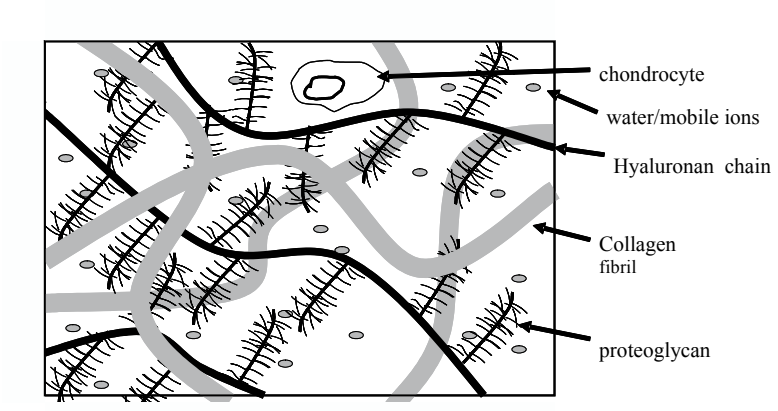

Figure 1. The main extracellular matrix constituents of articular cartilage. *The collagen fibrils form a network that gives the cartilage its tensile strength. In addition, proteoglycans are present within the collagen network to give the matrix a highly negative charge, resulting in a swelling pressure.*

Collagen type IX is crosslinked to type II collagen, located at the intersection of fibrils and may function as a connector molecule. Collagen type XI is present within the type II collagen fibrils and probably controls fibril thickness (5-8). Finally, type VI collagen represents only 1-2% of the total collagen content and is mainly found around the chondrocytes, providing multiple adhesion domains for cells and other matrix components. Proteoglycans are highly negatively charged macromolecules which attract water, resulting in 65 to 80% water content in the tissue. During loading, water is pushed out of the cartilage network until an equilibrium between loading forces and swelling pressure (caused by the negative charge density which gives resistance to water flow) is reached. Upon removal of loading, water (together with nutrients) is imbibed into the network, until the swelling pressure is balanced again by the resistance of the fibrillar collagen network.
Together, collagens and proteoglycans give cartilage the capacity to absorb and distribute loads and to present a low-friction surface. Hereby it protects the more rigid underlying bone and provides smooth articulation and bending of the joints during movement (9, 10).

1.1a Hyaline cartilage

Although only articular cartilage is found in the articular joints, other types of cartilage can be found throughout the body. There are three types of cartilage, defined by their structure and biochemical nature. Articular cartilage belongs to the most abundant type of cartilage; hyaline cartilage. Hyaline cartilage is avascular and 40% of its dryweight is composed of collagen type II, arranged in cross-striated fibers. Hyaline cartilage forms the embryonic skeleton and later it is found in the ossification center and at the lining of bones, where it is called the articular cartilage. The second type of cartilage is elastic cartilage. Elastic cartilage is similar to hyaline cartilage but contains elastin, forming elastic bundles. This type of cartilage is found in the ear and in tubes to keep the permantently open. The third type of cartilage is fibrocartilage. Fibrocartilage contains more collagen than hyaline cartilage and is found in areas requiring great tensile strength or though support, such as between intervertebral disks and tendon and ligament insertions at the bone (11).
Cartilage is also the tissue preceding bone and joint formation. During development of bones and joints, the skeleton is formed through condensation of mesenchymal precursor cells, which differentiate into chondrocytes (12). In the midsection of the skeletal elements the primary ossification center is formed, where the chondrocytes go through a process of proliferation, differentiation and maturation. Cartilage is resorbed and replaced by bone, forming the primary ossification center. In the epiphyseal cartilage at the end of the bone, a second ossification center is formed around birth. The layer of cartilage remaining between these two ossification centers forms the epiphyseal growth plate. This growth plate is responsible for the longitudinal growth of the bone (13).

Simultaneously to the formation of bone, at the determined ends of the skeletal elements, a thin band of mesenchymal cells, called the interzone, is formed. Cavitation at the site of the interzone leads to a physical separation of the skeletal elements and the formation of a synovial cavity (14). In addition, morphological changes in the interzone lead to the formation of articular cartilage along with intra-articular structures such as ligaments, menisci and the synovium (15, 16).

1.1b Chondrocytes

Chondrocytes are the only cells present in healthy articular cartilage. They are mesenchymal cells which differentiate during development and show no detectable proliferation after maturation. In addition, the collagen of articular cartilage has an estimated half life of about 200 years, indicating a slow turnover rate of cartilage (17, 18). Since cartilage is an avascular tissue, nutrition depends on diffusion from outside the tissue. Synovial fluid, present in the joint cavity, is an ultrafiltrate of plasma and diffuses into the cartilage during intermittent loading of the cartilage. The primary function of chondrocytes is to produce the cartilage extracellular matrix during growth and to maintain its integrity throughout life. For maintaining the extracellular matrix, the chondrocytes have to change their behaviour in response to external signals, such as alterations in the composition and organization of the matrix, including the presence of degraded molecules, and cytokines produced by other cells present in the joint (3, 19-22).
Although articular cartilage and the cartilage from the growth plate are both hyaline cartilage (as described above), the behaviour of the chondrocytes from the growth plate differs from the behaviour of the articular chondrocytes. In contrast to the articular chondrocytes, the chondrocytes from the growth plate are highly proliferative and later on become hypertrophic and will start to produce types of collagen found in bone and not in articular cartilage (such as collagen type I). This development of chondrocytes in the growth plate is regulated by systemic hormones, such as growth hormone, Insulin-like growth hormone, estrogen and androgen (23). In addition to these systemic hormones, locally produced growth factors such as the Indian Hedgehog (IHh)/parathyroid related hormone (PTHrP) feedback loop, fibroblast growth factors, (FGFs), vascular endothelial growth factor (VEGF), bone morphogenetic proteins (BMPs) and members of the Wnt-family play an important role in the proliferation and differentiation of the chondrocytes in the growth plate (24).

1.1c Collagens

Collagens are abundantly present throughout our body, as an extracellular matrix component of almost all connective tissues. There are several types of collagen; fibril forming collagens (e.g. type I, II, III, V, XI, XXIV and XXVII), fibril-associated collagens (e.g. type IX, XII and XIV), basement membrane-associated collagens (e.g. type

IV) and short chain collagens (25). A characteristic of all these collagens is the formation of triple helices, composed of three alpha chains, which can be either 3 identical (homotrimers) or 2 or 3 different polypeptide chains (heterotrimers). In order to form these triple helices, the α-chains must have a glycine (GLY) in every third position, resulting in a GLY-X-Y repeat. The X and Y positions can be filled by any aminoacid, but are often filled by a proline or hydroxyproline (26).
The most predominant collagen throughout the body is collagen type I. Collagen type I is the major component of most connective tissues, including skin, tendon, ligament, cornea and blood vessels. Two genes, COL1A1 and COL1A2 encode the collagen type I α1- and α-2 chain. In articular cartilage, collagen type II accounts for approximately 95% of the total collagen. Collagen type II is a fibril forming collagen and is a homotrimer of 3 α1-polypeptide chains, which are products of the COL2A1 gene (27).
Upon translation of the collagen genes, propeptide α-chains are formed. These procollagen chains consist of the collagen molecule with an N-propeptide and a C-propeptide attached. Upon transfer to the lumen of the endoplasmic reticulum, some proline and lysine residues are hydroxylated by prolyl-3-hydroxylase, prolyl-4-hydroxylase or by lysyl hydroxylases. There are three lysyl hydroxylase genes producing four proteins due to alternative splicing. These lysyl hydroxylases favour either helical or
non-helical regions of procollagen polypeptide chains as substrates (28). The hydroxylation of the proline and lysine residues is unique to collagens and takes place before the formation of the triple helix. Hydroxyproline is critical for the stabilization of the triple helix by formation of hydrogen bonds with other proline residues. In addition, some of the hydroxylysine residues undergo glycosylation by hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase (29). The role of glycosylation is not yet clear, but it is specific for collagen and appears to play a role in decreased susceptibility to proteolytic degradation (30).

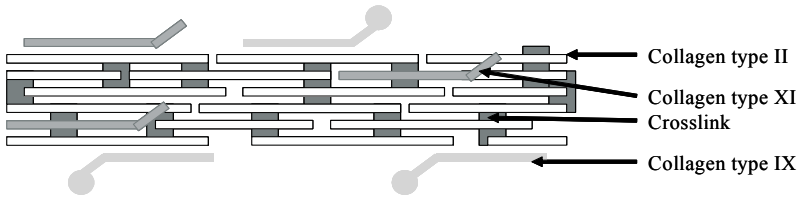

Figure 2 Schematic representation of the collagen network in articular cartilage*. Collagen type II fibrils are connected with collagen IX at the intersection of the fibrils. Collagen type XI is present within the collagen type II fibrils and controls fibril thickness.*

During and after these enzymatic modifications, a triple helix will be formed from the C-terminus to the N-terminus in a zipper-like fashion. Subsequently, the formed triple helices are secreted in the extracellular milieu by packaging in secretory vessels (31).

In the extracellular space the triple helices become less soluble after removal of the terminal propeptide regions by procollagen peptidases and subsequently self assemble into fibrils (32). The collagen fibrils are highly organized and, after their formation, stabilizing intermolecular pyridinoline crosslinks are formed. This collagen crosslinking is the final step in the biosynthesis of collagen. There are two routes through which this can occur; the allysine route or the hydroxyallysine route. In the allysine route, a lysine residue in the telopeptide is converted by lysyl oxidase into the aldehyde lysylallysine and in the hydroxyallysine route, a hydroxylysyl residue in the telopeptide is converted into hydroxyallysine aldehydes (33). The formed lysylallysine or hydroxyallisine reacts with a lysine or hydroxylysine residue in the triple helix to form di- or tri-functional crosslinks. Crosslinking through the allysine route normally occurs in the skin, while in bone, cartilage, tendon, ligaments and many other connective tissues, the hydroxyallysine route is most important for the formation of the crosslinking (34). The mature tri-functional crosslinks formed by this route are hydroxylysyl pyridinoline (HP), derived from three hydroxylysine residues, and lysyl pyridinoline (LP), derived from two telopeptide hydroxylysine residues and a lysine residue at the triple helical crosslinking site. The ratio of these crosslinks varies between the different types of collagen. In bone, the HP:LP ratio is much lower than in cartilage, in which HP is 30-50 times more present than LP (10, 34).

1.1d Proteoglycans

Proteoglycans consist of a core protein with highly sulphated glycosaminoglycan (GAG) chains covalently attached. There are seven different types of glycosaminoglycans found. Besides glycosaminoglycans, usually N- and O- linked oligosaccharides are also bound to the core protein. The core protein of the proteoglycans itself is attached by a link protein to a long chain glycosaminoglycan, hyaluronan, which is always present as a free carbohydrate chain.

Aggrecan is the most predominant proteoglycan in articular cartilage. The core protein of aggrecan consists of three globular domains (G1, G2 and G3) and two large extended regions (CS) are present for the attachment of the glycosaminoglycan chains (35). Aggrecan is highly glycosylated, mainly in chondroitin sulfate and keratan sulfate glycosaminoglycan chains, leading to the brush-like structure (*figure 3*). The amount of glycosylation is variable, resulting in a broad range of aggrecan compositions (36).

Stable aggregation of aggrecan is dependent on hyaluronic acid. Large proteoglycan aggregates are formed by the linkage of high amounts of aggrecan with hyaluronan by interaction of the two proteoglycan tandem repeats in the G-1 region of aggrecan with hyaluronan (37). The formation of these large aggregates fixes the proteoglycans within the collagen network, gives the tissue its stiffness to compression and contributes to its durability. In addition, the highly negative charge of the aggregate causes an osmotic force,

leading to a swelling pressure which enables the tissue to deform reversibly during loading (38, 39).

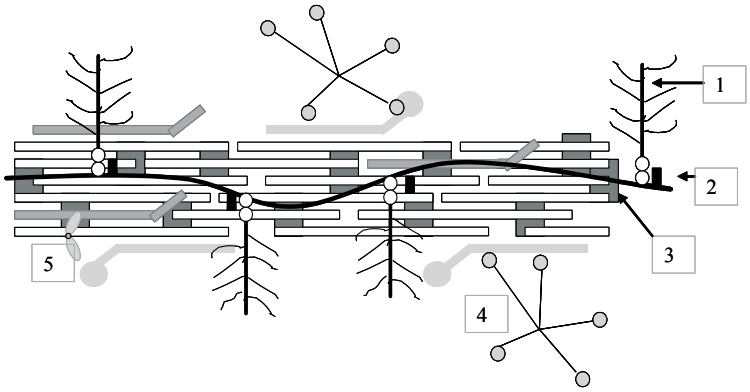

Figure 3. Schematic representation of proteoglycan structure within the collagen network. *The aggrecan coreprotein (1) is connected with a link protein (2) to hyaluronan (2) which fixes the complex within the collagen network. Other small proteoglycans such as COMP (4) and matrilin-3 (5) interact with the collagen network to provide stability and organisation.*

Besides aggrecan, some other proteoglycans are also present in articular cartilage. Although compared to aggrecan they hardly contribute to the mass of the matrix due to their small size, their molar amount is equal or higher than that of aggrecan (3). From several small proteoglycans the function is unknown, from larger proteoglycans such as decorin, fibromodulin and biglycan it is found that these small leucine-rich proteoglycans play a role in the interaction with collagen molecules. Cartilage oligomeric protein (COMP), decorin, matrilin-3, and fibromodulin interact with collagen type II and may play a role in stabilizing and organizing the collagen type II network (40-43) (*figure 4*). In addition, biglycan is concentrated in the pericellular matrix and may interact with collagen type VI (40).

1.1e Proteolytic cartilage degradation

There are two major proteolytic pathways for the degradation of ECM; the intracellular pathway in which proteins are hydrolysed by cysteine- and aspartate-proteases at low pH in lysosomes, and the extracellular pathways in which proteins are enzymatically digested by serine-proteases and metalloproteinases that act at neutral pH (44). This degradation of ECM plays a key role in tissue remodelling during normal processes such as embryogenesis and wound healing, but can also occur during pathologies such as tumour growth and invasion (45, 46).

Because of its unique composition, several classes of proteases are needed for the degradation of articular cartilage. Proteinases called aggrecanases, are specifically

responsible for the degradation of the major proteoglycan in cartilage, aggrecan (47). Helical collagen is resistant to most proteases, though collagenases are able to make a single cleavage, causing the triple helix to denature and make it more susceptible for further degradation by other enzymes such as gelatinases.

Aggrecanases are part of a subfamily of the A Disintegrin and Metalloproteinase family (ADAM) called a disintegrin and metalloproteinase with trombospondin motifs (ADAMTS). Most ADAM proteinases are transmembrane proteins and play a role in ectodomain shedding and activation of diverse cell surface proteins, such as growth factors and adhesion receptors (48). The members of the ADAMTS subfamily are secreted proteinases which in some cases bind to ECM. ADAMTSs can be involved in the cleavage of propeptide from collagen. There are several aggrecanases, ADAMTS -1, -4, -5, -8, -9 and -15, which are able to cleave aggrecan at 4 different sites. Aggrecanase-1 and -2 (ADAMTS-4 and -5 respectively) are the best studied aggrecanases and are able to cleave aggrecan at an additional site (49).

Collagenases are part of another important family involved in cartilage degradation: the matrix metalloproteinase (MMP) family of calcium dependent zinc endopeptidases (44, 50). The MMP family can be divided in subgroups: the collagenases (collagenases 1-3 also called MMP-1, -8 and -13), the gelatinases (gelatinase A and B, also called MMP-2 and -9), the stromelysins (stromelysin 1-4, also called MMP-3, -10, -11, -12), matrilysins (MMP-7, -26) and membrane-type MMPs (MT1- 6-MMP, also called MMP-14, -15, -16, -17, -24, -25) (50, 51). MMPs are produced as pro-enzymes that have to be activated by proteolytic removal of the N-terminal propeptide by components of the plaminogen activation system, by other MMPs, or by autolytic processes (52). This propeptide consists of 80 residues arranged in three α-helices and is responsible for the enzyme latency (50, 53). Besides this propeptide domain, the MMPs consist of two more domains; a catalytic domain that binds zinc and calcium ions and at the carboxy terminal end a haemopexin-like domain (54). The catalytic domains of MMP-2 and -9 are involved in interaction with various collagens and gelatins. The haemoplexin-like domain seems to play a role in substrate binding. Both the catalytic and the haemoplexin domain are required for the cleavage of triple helical collagen by collagenases, although other substrates can be cleaved by the catalytic domain alone (55).

Active MMPs can be bound by α2-macroglobulin and tissue inhibitors of MMPs (TIMP) resulting in inactivation of the MMP (56). In body fluids, α2-macroglobulin is primarily responsible for inactivation of MMP by entrapping it and subsequent clearing of the complex by the α2-macroglobulin receptor (57). TIMPs can not only bind active MMPs, but also proMMPs, thereby influencing the activation. Until now, four different TIMPs have been found, called TIMP1-4. All four TIMPs share a secondary structure of a three-loop N-terminal domain and an interacting three-loop C-subdomain, but they show distinctive structural features, biochemical properties and expression paterns. TIMP-1, -2

and -4 are present in soluble form, while TIMP-3 is only present tightly bound to the chondroitin sulphate of proteoglycans in the ECM (58). Although all four TIMPs are able to inhibit all active MMPs, the inhibition strength varies. TIMP-1 is a poor inhibitor of MT1-MMP, MT3-MMP, MT5-MMP and MMP-19. In addition, TIMP-3 is the only TIMP shown to inhibit members of the ADAM family, such as ADAM 12, ADAM 17 and the aggrecanases ADAM-TS4 and ADAM-TS5 (57). Together TIMPs regulate MMP activity by a 1: 1 binding of MMPs. Deregulation of the MMP: TIMP balance in favour of MMPs will lead to increased levels of activated MMPs and degradation of ECM.

1.2 The synovium

The joint space is confined by a joint capsula, which is lined by a highly specialized synovial membrane towards the joint cavity. The synovial membrane is divided in two distinct layers: the synovial lining (or intima) and the synovial sublining (or subintima). The lining layer is in direct contact with the intra-articular cavity. Under normal conditions it consists of only 1-2 cell layers of macrophage-like (type A) synoviocytes and fibroblast-like (FLS or type B) synoviocytes. The lining layer has an epithelial function: maintenance of the synovial cavity and the synovial fluid. Synoviocytes produce glycosaminoglycans (such as hyaluronic acid) and glycoproteins, to provide lubrication and nutrition to cartilage (15, 59). The fibroblast-like cells from the lining layer are, like other fibroblasts, able to produce extracellular matrix and have the potential to proliferate. The sublining consists of a collagenous extracellular matrix with scattered blood vessels and is relatively a-cellular containing fat cells and fibroblasts (1, 9).

1.2a Epithelium

The function of epithelial tissues is to cover or line body surfaces, forming the surface of the skin, the epidermis, the lining of body cavities (mesothelium) and the internal lining of the digestive system and glands. The epithelial layer is highly polarized; the apical surface is exposed to the external environment, while the basolateral surface is exposed to the internal milieu and is attached to a basement membrane, or basal lamina. This basement membrane consists of collagen type IV, glycoproteins such as laminin and fibronectin, and proteoglycans. Besides providing an adhesive surface, the basement membrane forms a restrictive barrier to the growth of the epithelial cells and serves as a semi-permeable barrier between tissue layers. The function of the epithelium is to form a barrier, and to regulate transport between the cavity it encloses and the adjacent tissue, by facilitating transport and secretion.

Another important feature of epithelial cells is their tight connexion and the presence of several junctions. The epithelial cells are connected by tight junctions, adherens junctions, desmosomes and gap junctions. Tight junctions or zonula occludens are located at the apical lateral region of the cells and seal the space between cells, thereby inhibiting

transport through intercellular spaces. The tight junctions are mediated by transmembrane claudins, occludins and scaffold proteins such as zonula occludens protein-1 (ZO-1) (60). ZO-1 is associated with intercellular actin of the cytoskeleton and signaling systems. Adherens junctions form a homophilic interaction between extracellular domains of E-cadherins, transmembrane calcium dependent adhesion proteins which are also intercellular linked to the actin cytoskeleton by α- and β-catenin (61). Desmosomes or macula adherins maintain the integrity of the epithelial unit by desmoglein and desmocollin, both cadherin molecules. Desmosomal plague proteins desmoplakin and plakoglobin link cell-cell adhesion molecules to the cytoskeletal keratin fibres (62). Finally, gap-junctions consist of connexins, protein subunits in the membranes of the two connecting cells. The gap junctions are communication junctions; the communication between two cells is facilitated by permitting small signaling molecules such as cAMP to diffuse through the connexin.

Synovial tissue shares some of the properties of epithelium. Presence of a fragmented basement membrane has been found in the synovial ling. Like epithelium, the lining layer of the synovium forms a polarized, one cell layer thick barrier between the joint space and the underlying tissue. In addition, the role of the fibroblast-like synoviocytes in the ultrafiltration (transport) and secretion of proteins resembles that of epithelial cells. In normal state, the function of the synovial tissue is to facilitate skeletal movement by the maintenance of a fluid-filled space around cartilage or tendon surfaces. The fibroblast-like synoviocytes are responsible for the excretion of factors such as hyaluronan into the synovial fluid, for clearance of intra-articular debris and regulation of immunological events (15, 59).

However, in the synovial lining only presence of gap junctions and desmosomes have been reported, no tight-junctions can be found by electron microscopy (59). The absence of tight junctions underlines the main difference between the synovial lining and epithelium: the discontinuous cell layer and the low amount of junctions present between synovial cells. Therefore, the synovial lining is epithelial-like rather than a real epithelium.

2 Joint diseases

The term arthritis is used for different kinds of joint diseases, which can be divided into three groups: arthritis of the connective tissues, inflammatory arthritis and non-inflammatory arthritis (63). During arthritis of the connective tissues, muscles, tendons and ligaments are affected. The most common inflammatory arthritis is rheumatoid arthritis, hallmarked by an inflamed synovium.

In non-inflammatory arthritis, inflammation is normally absent, or only minimally seen. The most common form of non-inflammatory arthritis is osteoarthritis, hallmarked by degradation of articular cartilage. In this thesis, the pathology of rheumatoid arthritis (RA) and osteoarthritis (OA) are further studied (*figure 4*).

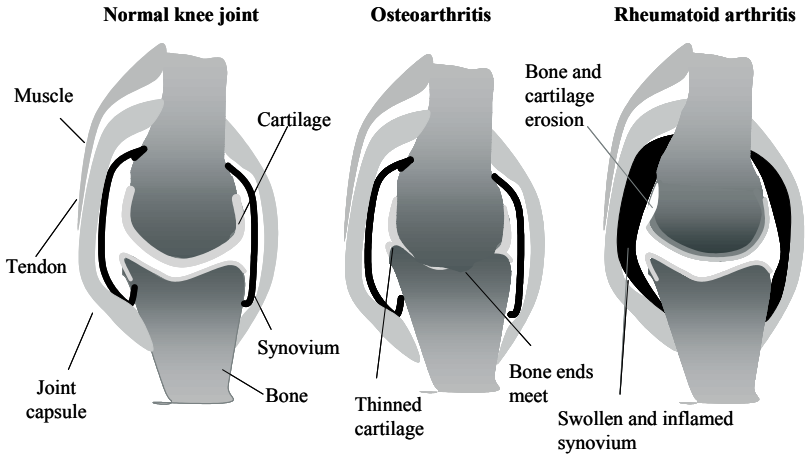

Figure 4. Schematic representation of a normal knee joint compared with an osteoarthritic knee joint and a rheumatoid knee joint.

2.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic crippling autoimmune disease with an incidence of 0.8-1 % of the world population (64). The disease affects 3 times more women than men and can occur at any age, but has its onset mostly between 30-50 years. RA is a systemic disease which is hallmarked by chronically inflamed joints. The synovial tissue becomes hypertrophic with proliferating lining layer and the sublining is infiltrated by cells from the immune system, forming a pannus tissue. This pannus tissue attaches to cartilage and bone, leading to cartilage destruction followed by bone erosion (65, 66).

Other parts of the body can also be affected; constitutional symptoms like fatigue, weight loss, fever and even disorders of heart, lungs, blood vessels, kidneys and nerve system can be found (67). Both genetic and environmental factor play a role in susceptibility and severity of RA. The genetic factor contributing for about a third of the genetic component is the Human Leukocyte Antigen (HLA) complex (also known as the major histocompatibility (MHC) complex). The Shared Epitope (SE) hypothesis has been formed around a concerved motif in the third hypervariable region of certain HLA-DR class II alleles, playing a role in the presentation of “arthritogenic” antigens to T-lymphocytes. Carriership for SE alleles increases risk for susceptibility and is associated with increased disease severity (68).

As for environmental factors consumption of coffee and smoking associate with increased risk for RA (69). Several proteins such as collagen type II and high mobility group box 1 (HMGB-1) are able to induce arthritis when injected into the joints of mice (70-72),

indicating that exposure to proteins from within the body could also be involved in disease onset.

2.1a Cell interactions in RA pathology

In RA pathology, many cell types interact at the site of cartilage degradation and inflammation. One of the cell types involved in RA pathology is the fibroblast-like synoviocytes (FLS or synoviocytes). Synoviocytes from RA patients are often described as cells with an altered phenotype or tumour-like or activated cells. The morphology of the synoviocytes changes into a spindle like shape and the cells start to produce matrix degrading enzymes, cytokines and growth factors (73). The activated phenotype of synoviocytes is maintained in culture even in the absence of cytokines or inflammatory cells, indicating their aggressive potential (74, 75).

A gene involved in the regulation of proliferation and apoptosis of synoviocytes is the p53 tumor suppressor gene. Wild type p53 suppresses proliferation and favours apoptosis. In RA patients, an overexpression of p53 has been found, combined with a decreased apoptosis rate. This can probably be explained by the fact that RA patients often show somatic mutations, inhibiting normal p53 function (76-80). In addition, expression of several oncogenes such as ras, raf, sis, myb, and myc are upregulated in synovial tissue, especially in synoviocytes (81, 82). Some of these proto-oncogenes are directly involved in the upregulation of MMP production, while others induce proliferation.

The most important MMP in RA is MMP-1 (collagenase 1), because it is produced in abundance by the synoviocytes from the inflamed synovium and it has the potency to degrade the collagens of the articular cartilage. Although it has previously been found that chondrocytes are the sole producers of MMP-13 (collagenases 3) (83), recently it has been found that after coimplantation of synoviocytes with cartilage in SCID mice, synoviocytes produce MMP-13 rather than MMP-1 at sites of cartilage degradation. In addition, it was found that upon culture in a 3D collagen gel or stimulation with interleukin -1β (IL-1β) or tumor necrosis factor α (TNF-α), synoviocytes are also able to produce MMP-13 (84, 85). Interestingly, stimulation of synoviocytes with IL-1β or TNF-α can induce expression of several MMPs, while these cytokines have no effect on TIMPs. Through this uncoupled regulation, IL-1β and TNF-α can alter the balance between MMPs and their TIMPs during RA, causing cartilage degradation. In total, expression of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13 and TIMP-1 has been found in synoviocytes of the lining layer of RA patients indicating that a broad range of collagenases, gelatinases and stromelysins is produced (86).

The pain and swelling of the joints of RA patients are associated with some features of synoviocytes. During RA, synoviocytes proliferate in the synovium, causing thickening of the synovial tissue, and associate with presence of excessive synovial fluid. This thickening and excessive fluid leads pressure on the surrounding tissues, causing the pain.

In addition, synoviocytes migrate to the cartilage, where they are found at sites of cartilage degradation (74, 87). Evidence for involvement of synoviocytes is also found in experiments; synoviocytes are shown to invade cartilage when coimplanted in SCID mice and to degrade cartilage *in vitro* (76). Invasiveness of synoviocytes can be influenced by many factors, such as TNF-α and IL-1β produced predominantly by macrophages, but also by other cell types.

Chondrocytes can have a stimulating effect on the invasive behaviour of synoviocytes from RA patients (RA synoviocytes). Increased invasiveness by RA synoviocytes is seen when seeded on fresh cartilage with viable chondrocytes, compared to RA synoviocytes seeded on stored cartilage with less viable chondrocytes. In addition, cartilage degradation was also inhibited when RA synoviocytes were seeded in 3-D collagen sponges with chondrocytes whose protein production was inhibited by cycloheximide (88). Though cell contact seems to be very important, chondrocytes can, depending on their viability, degrade cartilage when separated from synoviocytes, under the influence of IL-1β, a soluble factor from synoviocytes and macrophages (86, 89, 90).

Expression of aggrecanases-1 and -2 (ADAMTS-4 and -5), enzymes able to cleave aggrecan molecules and free them from the collagen network, was found constitutively in RA, OA, and healthy synoviocytes and in chondrocytes. Interestingly however, in synoviocytes, TNF-α and TGF-β were able to induce an increase in ADAMTS-4 production, while IL-1β had only minimal effect. None of the cytokines had an effect on ADAMTS-5 levels in synoviocytes (91). In contrast, the activity of ADAMTS-4 produced by chondrocytes was increased by a combination of oncostatin M (OSM) and IL-1β and ADAMTS-5 expression by chondrocytes was inducible by IL-1β alone (92-94). In addition, the *in vivo* expression of ADAMTS-5 in cartilage was much higher than that of ADAMTS-4 (93). This indicates that regulation of cartilage degrading enzymes is regulated differently in synoviocytes and chondrocytes, so activation of both cell types might be involved in cartilage degradation.

Besides interacting with each other, both synoviocytes and chondrocytes can interact with cells from the immune system such as monocytes, macrophages, and T-lymphocytes. In the cell interactions in RA pathology, several cytokines play a role; in synovial tissue and –fluid, levels of tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor growth factor β (TGF-β) are increased (95-98).

2.1b Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is a process normally involved in development of the embryo. It facilitates formation of a three-layered embryo during gastrulation by producing the mesenchyme that condenses to form the middle layer called mesoderm and the inner layer called endoderm. Furthermore, EMT and the reverse

process mesenchymal to epithelial transition (MET) underlie organogenesis, particularly in the heart, musculoskeletal system, craniofacial structures, and peripheral nervous system (99). Both processes have emerged as a fundamental principle for reprogramming of gene transcription and as a major determinant of stem cell fate in development and in tissue homeostasis. EMT can occur in epithelial cells of all embryologic origins and is phenotypically characterized by the loss of cell-cell contact, loss of polarity, change to a spindle-cell morphology, and the acquisition of an invasive motility. Processes associated with these changes are the disassembly of the cell junctions, induction of vimentin, and rearrangement of the actin cytoskeleton (100-102).

Besides a role in embryology, EMT also plays a role in pathologies during such as carcinomas and fibrotic processes (102-105). EMT can be induced by degradation of the basement membrane by MMP-2 and –9, or by secreted inducers such as TGF-β, basic fibroblast growth factor (FGF), endothelial growth factor (EGF), (IGF-II), and (HGF). The best described inducer of EMT is TGF-β. TGF-β- like activins and BMPs bind to serine/threonine kinase I and II receptors. After a ligand induced heteromeric complex formation between TGF-βRI and TGF-βRII, receptor activated Smads (Smad-2 and –3) are phosphorylated. These phosphorylated Smads bind to a common mediator Smad, (Smad 4) in the cytoplasm and are subsequently translocated to the nucleus. In the nucleus, the Smad complexes interact with gene regulating motifs of target genes (106). Another member of the TGF-β superfamily, BMP-7 (or OP-1) also induces a Smad signaling pathway by binding to ALK2 and -3. BMP-7 signalling consists of receptor activated Smads 1,-5, and –8. After phosphorylation of these Smads they also partner with Smad 4 before translocation to the nucleus (106). BMP-7 signaling leads to upregulation of E-cadherin, and thereby to inhibition of EMT and induction of MET. *In vitro*, BMP-7 can reverse TGF-β induced EMT in a mouse epithelial cell line and even *in vivo* evidence for reversal of chronic renal injury by systemic administration of BMP-7 has been shown (107).

2.1c Change of phenotype of fibroblast-like synoviocytes during RA

The changes that occur in the synovial lining of RA patient somewhat resembles the changes that occur in tumour metastasis and fibrosis. During both tumor metastasis and fibrosis, alteration in the phenotype of epithelial cells by epithelial to mesenchymal transition (EMT) is involved.

During tumor metastasis, a similar change in phenotype of the cells is seen. Cells change from epithelial cells into large spindle-shaped cells with migratory and invasive properties. These properties are also the most important hallmarks of EMT.

Fibrosis is the de-regulation of normal wound healing. During normal wound healing, re-epithelialisation occurs by replication and movement of epithelial cells. Fibroblast and myofibroblasts are responsible for the *de novo* formation of extracellular matrix. In

addition, myofibroblasts are responsible for the contraction of the tissue. Myfibroblasts are formed from fibroblasts, or through EMT from epithelial cells. They are connected to each other with gapjuntions and in addition have a contractile apparatus that contains bundles of actin microfilaments (also called stress fibres) with associated contractile proteins. The actin fibres terminate at the surface of the cells in the fibronexus, which is a specialized form of adhesion complex linking intracellular actin with extracellular fibronectin fibrils by using transmembrane integrins (108). Mechanical stress, TGF-β1, and ED-A fibronectin are the main factors for development of myofibroblasts from fibroblast of the granulation tissue or from epithelial cells through EMT (109). After production of ECM and contraction and closing of the wound in the normal process, a dismantling of the cellular apparatus occurs by apoptosis or differentiation into a quiescent form and restoration of the normal tissue structure (110, 111). During fibrosis, abnormal wound healing occurs by persistence of myofibroblasts and an excessive deposition of extracellular matrix proteins. This eventually leads to loss of function of the organ (112). During fibrosis, not only development of myofibroblast by EMT is induced by profibrotic cytokines, but also production of extracellular matrix proteins, especially collagen type I and III by myofibroblasts can be stimulated by several profibrotic cytokines, of which TGF-β is believed to be the most potent (113).
During RA, both alterations in synoviocyte characteristics, often described as acquisition of tumor-like characteristics, are seen as well as accumulation of extracellular matrix (114-116). Therefore it is likely that during RA, synoviocytes undergo a process similar to tumor and fibrotic cells.

2.2 Osteoarthritis

Osteoarthritis (OA) is one of the most common forms of arthritis (117, 118). OA is a degenerative joint disease that has a major impact on cartilage function. Although cartilage degradation is the primary effect of OA, the pain observed by OA patients is caused by the secondary effects, since cartilage lacks innervation (119). The secondary effects of OA are synovitis and formation of osteophytes by elevation of bone proliferation, causing joint deformity which in turn leads to distension of the joint capsula, muscle spasms, and damage to soft tissue (120-123).
In OA disease onset, many risk factors are involved: systemic factors (genetics, oestrogen use, and bone density) and local biomechanical factors (muscle weakness, obesity, and joint laxity). The most important risk factors however, are female sex, obesity, joint trauma, and age (117, 118, 124). During aging, several alterations in cartilage mechanisms occur that can make the cartilage more susceptible to OA. Some of these changes are the reduction of chondrocyte response to growth factors, altered synthesis of extracellular matrix proteins, and accumulation of advanced glycation endproducts (AGEs) (98, 125-128).

Among many other features of OA, increased levels of MMPs and cytokines such as IL-1β and TNF-α are found in synovial fluid (129, 130). In the early stage of OA, before inflammation of the synovium, production of MMPs by chondrocytes is present. After inflammation of the synovium has initiated, MMP production by synoviocytes has also been found, although at the site of cartilage-synovium contact, MMP-13 (the most potent MMP to degrade collagen type II) produced by chondrocytes is predominantly found. Besides MMP13, two other collagenases are found in increased levels in OA articular cartilage; collagenase 1 (MMP-1) and collagenase-2 (MMP-8), which are also able to degrade the collagen type II in the cartilage. In addition, gelatinases MMP-2 and MMP-9 and stromelysin-1 (MMP-3) are also increased in OA cartilage. Although MMPs can induce aggrecan release *in vitro*, aggrecanases are able to cleave aggrecan at physiolocially relevant sites (131), resulting in the release of C-terminal fragments that have been identified in synovial fluid. Therefore, upregulation of aggrecanases by cytokines such as IL-1β and TNF-α (present in high levels in OA synovial fluid) plays an important role in degradation of proteoglycans in cartilage (94).
Production of several MMPs is upregulated by IL-1β, although this is often dependent on the layer of cartilage. The superficial layers of the cartilage are already stimulated at low levels of IL-1β, while the deeper layers need a high level of IL-1β (132). In the superficial cartilage layers the degradation of collagen is most pronounced (21).
Although systemically only evidence for cartilage degradation is found, the chondrocytes of the deeper layers of cartilage also show a compensation mechanism. To compensate for the collagen and aggrecan degradation by MMPs and ADAMTSs, the chondrocytes show increased collagen and aggrecan production (133). In the fibrillar regions of the degenerated cartilage, hypertrophic chondrocytes are found which can produce collagen type X (134-136). In addition, collagen type III and VI, other non-cartilage collagens, are also produced, leading to an alteration in matrix properties and inefficient cartilage (137).

3 RAGE

The receptor for advanced glycation end products (RAGE) is a multi-ligand member of the immunoglobulin superfamily of cell surface molecules. RAGE is composed of three extracellular domains, the V domain and two C domains, followed by a hydrophobic transmembrane spanning domain and a short acidic carboxyl-terminal cytoplasmic tail (*figure 5*). The cytoplasmic tail is essential for the intracellular transduction pathway (138, 139). For binding of RAGE ligands, such as advanced glycation endproducts (AGEs), amyloid fibrils, S100/calgranulins, and high mobility group box (HMGB-1), the V-type domain is critical.
The RAGE gene is located on chromosome 6p2.13 in the major histocompatibility complex (MHC) locus in the class III region (140). Several splice variants are expressed, encoding for the isoforms that lack the N-terminal V-type domain or the C-terminal

transmembrane domain. The N-terminal truncated form is unable to bind ligands, but overexpression in cells does not lead to change in signalling when the full-length RAGE is present. In contrast, the C-terminal truncation encodes a soluble, secreted RAGE (sRAGE) which is able to bind its ligands (139, 141, 142). This soluble form has been found in serum of humans and is able to capture ligands in this serum. A fourth isoform for RAGE is obtained by the deletion of the intracellular cytosolic tail, resulting in a truncated form which is present on the cell surface. It is able to bind its ligands, but is unable to induce the signalling pathway. This form of RAGE is called dominant negative because its presence on a cell blocks RAGE signalling, even in the presence of the full length receptor (138, 143).

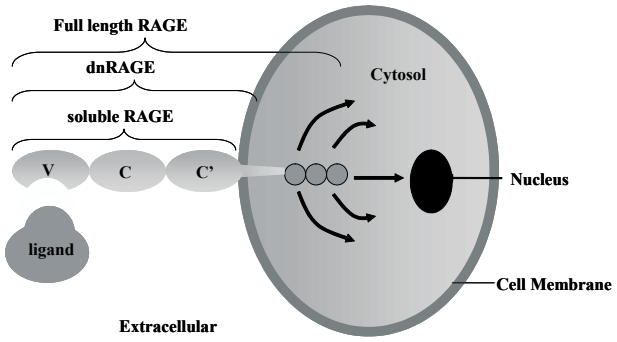

Figure 5. Schematic representation of RAGE*. RAGE is built up of three extracellular domains; two C domains and a ligand binding V domain. Furthermore, RAGE has an transmembrane domain and an intracellular domain, responsible for activating downstream signalling pathways.*

In addition to different isoforms, several polymorphisms in the RAGE gene have been found. Most of the nucleotide changes did not result in functional amino acid changes, except for the RAGE G82S polymorphism. This polymorphism gives a nucleotide change from a G to an A, resulting in an amino acid change from a glycine to a serine at position 82 in the ligand binding domain of the receptor (144). A functionality study of this polymorphism demonstrated that cells bearing a heterozygous polymorphism are already more responsive to its ligands, suggesting the possible relevance of the polymorphism (145).

3.1 RAGE ligands

Accumulation of RAGE ligands, leading to upregulation of the receptor and sustained cell activation, plays a role in a number of pathological processes such as renal failure, amyloidosis, diabetes, and inflammatory disorders (*table I*) (139, 146).

3.1a AGEs

Non-enzymatic glycation is a common posttranslational modification of proteins caused by reducing sugars. The spontaneous condensation of reducing sugars with free amino groups in lysine or arginine residues on proteins, lipids, and nucleic acids leads to the formation of a reversible Schiff-base (an unstable glycosylamine). This Schiff-base is subsequently stabilized by Amadori rearrangement (forming ketosamines). Next, Maillard or browning reactions (a series of reactions leading to the formation of polymeric,

Table 1. Increased levels of RAGE ligands are involved in several pathologies

Diabetic complications	AGEs S100/calgranulins
Amyloidoses	β-sheet fibrils AGES S100/calgranulins
Immune/inflammatory disorders	AGEs S100/calgranulins Amphoterin/HMGB-1
Tumor biology	Amphotherin/HMGB-1

browning or fluorescent compounds) convert the initially formed intermediate products into advanced glycation endproducts (AGEs) (98, 147). Glucose has the slowest reaction rate while intracellular sugars, such as glucose-6-phosphate and fructose, form AGEs at a faster rate (148-150). Besides this classical pathway of AGE formation, more recently it has been found that AGE formation can also be initiated by metal-catalyzed glucose auto-oxidation, as well as by lipid peroxidation (98). When glycation is accompanied by oxidation, glycoxidation products such as pentosidine and N^{ε}-[carbosymethyl]-lysine (CML) are formed (149). The diversity in reaction pathways results in a variety of chemical structures of AGEs. Some AGEs are adducts to proteins, while many others present protein-protein crosslinks. Once they are formed, AGEs cannot be removed from the protein and therefore AGEs only leave a tissue when the protein involved is degraded. Since the rate of AGE accumulation is largely determined by the rate of protein turnover (18), this low turn over results in an abundant accumulation of AGEs in long lived proteins (10, 128).

The level of AGEs increases with age, but also increases in an accelerated rate in the presence of hyperglycemia (for instance in diabetic patients) and oxidative stress (which occurs during inflammation) (148, 151, 152). The increase of AGE crosslinks leads to

increased stiffness of the protein matrix, thereby altering the function. In addition, increase of AGE crosslinks increases the resistance of the matrix against proteolytic removal, which in turn affects the process of tissue remodelling (153). The increase of AGE crosslinks does not only have biochemical and biomechanical effects, direct effects on cell metabolism within the matrix can be mediated by several receptors such as RAGE.

3.1b HMGB-1

High mobility group box 1 (HMGB-1) was previously called HMG-1 or amphoterin and is an intranuclear factor with multiple functions. Intranuclear, HMGB-1 plays a role in stabilizing nucleosome formation and in DNA-folding, thereby facilitating gene transcription, recombination and repair (154). Furthermore, HMGB-1 also plays a role in cell replication, cell migration and tumor growth (143, 155). Extracellular functions of HMGB-1 have also been found; multiple observations suggest HMGB-1 can act as a potent cytokine (156-158). HMGB-1 can be found extracellular after necrosis or after activation of monocytes and macrophages. Necrotic cells release nuclear HMGB-1 passively into the extracellular milieu (159), but HMGB-1 can also be actively secreted by activated monocytes and macrophages (143, 156, 160, 161). Extracellular HMGB-1 can bind to RAGE on monocytes and macrophages after which it induces expression of cytokines such as IL-1β, TNF-α, and IL-6 by these monocytes and macrophages (161).

3.1c S100/calgranulins

S100/calgranulins comprise a family of about 20 calcium binding polypeptides. The small acidic proteins contain two distinct EF hands (containing an 'E' and a 'F' helix joined by a Ca^{2+} binding loop); one located at the N-terminus connected to the second, a classical Ca^{2+}-binding EF hand, by a central hinge region. Intracellularly, S100 proteins act as Ca^{2+}-signalling or –buffering proteins and are involved in several cellular processes such as cell growth and motility, cell cycle regulation, transcription, and differentiation. Extracellularly, the S100 proteins act in a cytokine-like manner by binding to RAGE (162-164). Because S100 proteins can occur in homo- and heterodimers and in oligomers, and show cell and tissue specific expression paterns, their function is diverse. Several members of the S100 protein family are implicated to be involved in diseases of the heart (ischemia, end stage heart failure, hypertrophy), diseases of the central nervous system (Alzheimer, Down syndrome, multiple sclerose), cancer (neoplasias) and inflammatory disorders (host response, chronic bronchitis, cystic fibrosis, RA) (162, 165-170).

S100 protein is found only faintly in normal articular cartilage, but in higher levels in active chondrocytes, such as the hypertrophic chondrocytes representing hypertrophic chondrocytes in the perivascular areas of the neonatal articular cartilage and in the deep zone of the infant articular cartilage (171). In articular chondrocytes, S100 protein is involved in changes into chondroic tissue. It plays a role in healing of the tissue for it is

able to induce a change from fibroblast-like cells to chondrocyte-like cells, causing chondroid metaplasia (172).

3.2 RAGE ligands in RA

Several animal studies have indicated a possible role for RAGE in the onset and severity of arthritis. Blockade of RAGE by addition of soluble RAGE in a mouse model for collagen induced arthritis not only suppresses cytokines levels but also suppresses clinical and histological evidence of arthritis, showing that blockade of RAGE suppresses disease severity in these mice (145). Administration of the RAGE ligand HMGB-1 in several mice strains resulted in arthritis in 80% of the animals, indicating that increased RAGE ligands could play a role in disease onset (70). In-line with these findings, increased levels of RAGE ligands have been found in RA patients and correlate with disease severity.

Higher levels of HMGB-1 are found in synovial fluid of RA patients compared to OA patients (161). The effect of RAGE activation through HMGB-1 on synovial macrophages has recently been studied by Pullerits *et al* (70) and Taniguchi *et al* (161). Extracellular HMGB-1 stimulates macrophages to produce proinflammatory cytokines and chemokines (e.g. interleukin 1 (IL-1) α and β, and tumor necrosis factor (TNF α) (70, 154, 156, 161).

Not only HMGB-1 levels are increased in RA, but also levels of pentosidine, one of the AGEs (described above) is increased in RA serum, urine, synovial fluid, and synovial tissue (173-176). In addition, levels of another RAGE ligand, S100 calgranulins are increased in RA (162).

Similar to chondrocytes and macrophages, synoviocytes involved in the pathology of RA express RAGE (177). Therefore, RAGE activation by increased levels of its ligands in RA might not only play a role in altered matrix synthesis by chondrocytes and inflammatory processes mediated by macrophages, but also in activation of synoviocytes.

3.2a RAGE G82S polymorphism in RA

In addition to increased levels of RAGE ligands, polymorphisms in the RAGE gene associated with RA phenotypes indicate a role for RAGE in RA pathology. A gain of function polymorphism of RAGE in the ligand-binding domain, a change form glycine to serine located at position 82, has a higher prevalence in RA than healthy controls. Hoffmann *et al* found that compared to cells bearing the 82G allele, cells bearing the 82S allele show increased binding and cytokine production upon ligation with S100/calgranulins (145). This again suggests RAGE might play an important role in proinflammatory mechanisms in immune and inflammatory diseases such as RA. However, the RAGE gene is located on chromosome 6 in the HLA region (178) and is in linkage disequilibrium with DRB1*0401, one of the Shared Epitope (SE) alleles. After correction for association with this allele, the correlation between the RAGE 82S

polymorphism and RA was lost, although possibly due to the small number of patients and controls bearing the 82S polymorphis; a type II error can not be excluded.

3.3 RAGE ligands in OA

One of the most important risk factors for OA is aging. A prominent feature of aging is the accumulation AGEs in long-lived proteins, like cartilage collagens (128, 179). As described above, AGEs are formed during non-enzymatic glycation of arginine and lysine residues by reducing sugars. Since AGEs can only be removed when the whole protein is removed, they accumulate in the long-lived proteins. Accumulation of AGEs in cartilage leads to an alteration in mechanical properties and in cartilage metabolism; cartilage stiffness increases substantially with increasing AGE levels and matrix synthesis by articular chondrocytes becomes impaired (180, 181). This alteration in matrix synthesis might be mediated by receptors for AGEs. Several receptors have been identified: scavenger receptors type I and II, oligo saccharyl transferase 48 (AGE-R1), 80 K-H phosphoprotein (AGE-R2), galectin-3 (AGE-R3) and the receptor for advanced glycation endproducts (RAGE) (98).

In addition to a possible role for AGEs in OA pathology, S100 proteins might also be involved. On normal articular chondrocytes, several S100 proteins are faintly present. In OA however, it is found that expression of some S100 proteins is increased (182-184). It is not clear exactly what function S100 proteins have in OA pathology. S100A11 was found to induce hypertrophy by signalling through RAGE, while S100B and S100A4 were found to induce MMP-13 production by articular chondrocytes.

This latter effect was also found for HMGB-1; stimulation of chondrocytes with HMGB-1 leads to increased MMP-13 production, indicating increased cartilage degradation (182).

Aim

The aim of this thesis is to study cellular processes in joint pathology/physiology. To this end an artificial joint model was developed. The RAGE pathway of cellular activation was studied for its role in joint pathophysiology. Moreover, the cellular activation state of the cells in the model was analysed.

Outline of this thesis:

Studies of cartilage degradation in OA and RA often focus on a specific cell type. In OA, chondrocytes are studied, while in RA synoviocytes are used. However, several indications have been found that cell interaction between synoviocytes and chondrocytes plays an important role in both OA and RA pathology. Since adult human chondrocytes are difficult to obtain and the use of bovine chondrocytes is widely accepted and applied**,** bovine chondrocytes are combined with human RA synoviocytes to study cell interactions in cartilage degradation in ***chapter 2***.

In addition to increased AGE levels in OA articular cartilage, RAGE expression has been found on both chondrocytes and synoviocytes. The effect of RAGE activation by AGEs is described in ***chapter 3***. In chondrocytes, the effect on cartilage degradation was studied by measuring GAG release after incubation with glycated albumin. In addition, the effect of glycated albumin on the mRNA expression of collagen type II and MMP-1 was studied. In synoviocytes, invasiveness through a Matrigel matrix and production of (pro) MMP-1 were taken as measures for change to an activated state after stimulation with a glycated matrix or glycated albumin.

The effect of the RAGE G82S polymorphism on susceptibility for RA is described in ***chapter 4***. Although it was previously described that the 82S polymorphism might have an effect on susceptibility for RA, our study shows that RAGE 82S does not correlate with RA independently of HLA DRB1*0401.

Several RAGE ligands have increased levels in synovial fluid from RA patients. In addition, synoviocytes show expression of RAGE. In ***chapter 5*** it is described how addition of HMGB-1 and glycated albumin is able to stimulate invasiveness of RA synoviocytes in the Matrigel invasion model by activation of RAGE.

Epithelial cells are cells that line the outside of the body or an internal cavity. Their function is to form a barrier and facilitate exchange of proteins and fluid. In several pathologies such as tumor proliferation/migration and fibrosis, an alteration in phenotype and behaviour of the affected cells is described. This process called epithelial to mesenchymal transition can occur in all types of epithelial cells, regardless of their origin. In ***chapter 6***, characteristics of the synovial lining and epithelium are compared. In addition, it is described that RA synoviocytes have undergone a process resembling EMT/fibrosis.

A general conclusion of this thesis can be found in ***chapter 7***, where the results of the individual studies are summarized and discussed.

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Chapter 2

Fibroblast-like synoviocyte-chondrocyte interaction in cartilage degradation

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Abstract

Introduction*: In vitro models for joint diseases often focus on a single cell type, such as chondrocytes in osteoarthritis (OA) or fibroblast-like synoviocytes (synoviocytes) in rheumatoid arthritis (RA). However, these joint diseases affect the whole joint and interaction between chondrocytes and synoviocytes may play an important role in disease pathology. The current study was designed to study the use of the alginate recovered chondrocyte method as a model for cartilage degradation and to study interaction between chondrocytes and synoviocytes.*

Methods*: Bovine chondrocytes were cultured in alginate beads for 1 week; subsequently chondrons were retrieved and seeded into transwells. Every two days cartilage-slices were analysed for proteoglycan content (colorimetric, Blyscan GAG kit), collagen content (HPLC) and collagen HP and LP crosslinking (HPLC). For degradation experiments, monocultures of cartilage-slices labelled with ^{35}S and cocultures with synoviocytes were stimulated with IL-1β or TNF-α. After 7 days ^{35}S release was taken as a measure of cartilage degradation.*

Results*: After biochemical analysis, three week old cartilage-like slices were chosen to perform cartilage-degradation experiments. Synoviocytes were able to induce cartilage degradation only in the presence of living chondrocytes. In addition, the cytokines interleukin 1 (IL-1β) and tumor necrosis factor (TNF-α) were only able to induce cartilage degradation by chondrocytes, not by synoviocytes.*

Conclusion*: These data indicate that the alginate recovered chondrocyte method provides a novel model for cartilage degradation in which the interaction between synoviocytes and chondrocytes can be studied.*

Introduction

Rheumatoid arthritis (RA) is characterised by hyperplasia and inflammation of the synovial tissue, which forms the so-called pannus tissue and degradates the articular cartilage at the cartilage-pannus junction, leading to loss of joint function and disability (1;2). Fibroblast-like synoviocytes (synoviocytes) of the synovial lining layer are thought to play a key role in the hyperplasia of the synovial tissue and the degradation of the cartilage (3;4). During inflammation, immune cells diffusing into the synovial fluid (SF) and synovial tissue produce inflammatory mediators that are able to stimulate cartilage degradation by synoviocytes and chondrocytes and that cause the hyperplasia of the synovial tissue. For example, macrophages produce matrix metalloproteinases themselves, but are also an important source of interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), cytokines which stimulate matrix metalloproteinase production by chondrocytes and synoviocytes (5;6). Although the contribution of the different cell types in joint homeostasis and pathology is generally recognized, not many studies address the

interaction between the cell types involved. To study RA pathology, both *in vivo* and *in vitro* models are applied to elucidate pathways and to study treatment efficacy. Of the *in vivo* models, the most common models are collagen induced arthritis (CIA) in rats and mice, adjuvant-induced arthritis (AIA) in rats, and the transplantation of human tissues in severe combined immune deficient (SCID) mice (7-9).

In vitro models are potentially more suitable and convenient to study cell-cell interactions. Most *in vitro* disease models that are used to study RA disease mechanisms focus on synoviocytes or the complete synovial tissue when RA is studied. To study synoviocyte invasiveness, Transwell invasion systems with Matrigel^{TM} (collagen type IV) or Invitrogel (collagen type I) are used. One of the downsides of these models is that the Matrigel and Invitrogel are both composed of different components than cartilage, which primarily contains collagen type II and proteoglycans along with minor quantities of other proteins. It is desirable to use a matrix which resembles real cartilage because different mechanisms are involved in the degradation of different proteins or tissues. Cartilage type II collagen degradation for example, is mediated by a different panel of proteases than type I collagen in bone or skin. Therefore, to understand the mechanism involved in cartilage degradation, a matrix consisting of the same components as articular cartilage should be used. Another important aspect of cartilage degradation is the presence or absence of chondrocytes. The interaction between chondrocytes and synoviocytes may play an important role in disease pathology, since chondrocytes are able to degrade their surrounding cartilage. In addition, both chondrocytes, synoviocytes, and also infiltrating macrophages are able to produce inflammatory mediators (e.g. IL-1β and/or TNF-α) and can be activated by these cytokines (5;6;10;11).

In the present study we investigate the use of cartilage-like slices obtained by the alginate recovered chondrocyte (ARC) method in an *in vitro* model for cartilage degradation. To validate the model, we investigated the interplay between chondrocytes and synoviocytes. In addition, the effect of IL-1β and TNF-α, the most important cytokines produced by macrophages, on the degradation of the cartilage-like slices by chondrocytes and synoviocytes was studied.

Material and methods

Chondrocyte isolation and culture

Chondrocytes were isolated from the metacarpophalangeal joints of 6 month old calves. Cartilage was harvested and digested with 0.2% pronase for one hour, followed by overnight digestion with 0.025% collagenase. Cells were separated from the undigested tissue using a 200 μm filter (Braun Medical, Oss, the Netherlands) and cultured in 1.2% low viscosity alginate (Keltone, LV, Kelco) at $4x10^6$ cells/ml for 7 days in a 1:1 mixture of DMEM (GibcoBRL, Paisley, UK) and HAM's F12 (BioWhittaker, Versiers, Belgium), supplemented with 0.28 mM ascorbic acid (Sigma-Aldrich, Steinheim, Germany), 100

IU/ml penicillin (Biowitthaker, Versiers, Belgium), 100 µg/ml streptomycin (BioWitthaker) and 20 % foetal calf serum (FCS, GibcoBRL). After one week, beads were dissolved in sodium citrate (55 mM in 0.9 % NaCl) for 20 minutes. Chondrocytes with their cell associated-matrix were centrifuged for 10 minutes at 110 g and resuspended at a concentration of 300 beads per 2.5 ml of medium. 0.5 ml of cell suspension was transferred to the inner compartment of a 12 mm Transwell. 1.5 ml of culture medium was added in the outer compartment. This procedure results in the formation of cartilage-like slices during three weeks of culture and twice weekly slices (n=3 per time point) were frozen at -20 ºC for biochemical analysis. Medium was refreshed three times per week.

Proteoglycan measurement

Cartilage-like slices were digested for 24 hr at 56 ºC in papain buffer containing 3% v/v papain, 5 mM cysteine HCl, 50 mM EDTA and 0.1 M sodium acetate, the buffer was adjusted to pH 5.5. The amount of sulphated glycosaminoglycans (GAGs) was determined in an aliquot (1 µl) of the papain digest by dimethylene blue staining (Biocolor Ltd, Belfast, N. Ireland), using chondroitin 4-sulfate as a standard.

Collagen content and crosslinking

A second aliquot (500 µl) of the papain digests was hydrolyzed by addition of 500µl of 12 M HCl at 110°C for 20-24 h for cross-link and amino acid analysis. Hydrolyzates of the papain digests were dissolved in water containing the internal standards pyridoxine (10 µM; Sigma) and homoarginine (2.4 mM; Sigma). For collagen cross-link analysis (hydroxylysyl pyridinoline (HP) and lysylpyridinoline (LP)), samples were diluted 100-fold with 50% acetic acid and analyzed by HPLC (12). For amino acid analysis, an aliquot of the cross-link samples was diluted 25-fold with 0.1 M borate buffer (pH 11.4), derivatized with 9-fluorenylmethyl chloroformate (Fluka) and analyzed by HPLC (13). HP, LP, and hydroxylysine (Hyl) are expressed as mol per mol collagen, assuming 300 hydroxyproline (Hyp) residues per triple-helical collagen molecule (12).

Isolation and propagation of rheumatoid fibroblast-like synoviocytes

Synoviocytes were obtained at joint replacement surgery or synovectomy from RA patients after informed consent. Tissue was collected in sterile phosphate buffered saline (PBS). Fat and connective tissue were removed and tissue was digested with collagenase (CLS2, Worthington Biochemical Corporation) for 2-3 hours at 37 ºC. Cells were then separated from the undigested tissue using a 200 µm filter (B-Braun Medical, Oss, the Netherlands) and cultured in 162 cm^2 culture flasks (Costar, Cambridge, NY, USA) with Iscove's Modified Dulbecco's medium (IMDM; Biowitthaker, Versiers, Belgium) supplemented with glutamax (Biowhittaker), 100U/ml penicillin and streptomycin (Biowitthaker) and 10% foetal calf serum (FCS, GibcoBRL) in a humid atmosphere of 5%

CO_2 in air at 37 ºC. Upon reaching confluence, cells were detached with 0.25% trypsin and split in a 1:3 ratio. For all experiments 3^{rd}-5^{th} passage synoviocytes were used. Using light microscopy >95 % of cells were judged to be synoviocytes.

Radioactive labelling with sulphate

To establish a sensitive method for the detection of cartilage degradation of the cartilage-like slices, the matrix was labelled with ^{35}S sulphate (Amersham, Braunschweig, Germany). 0.25 µCi of ^{35}S in sulphate solution was added per ml medium during the first week of culture in Transwells. The following two weeks culture medium without the radioactive tracer was used.

Coculture of rheumatoid fibroblast-like synoviocytes with cartilage-like slices

Isolated RA synoviocytes were added on top of the cartilage-like slices in the inner compartment of the Transwells in a volume of 0.5 ml of serum-free DMEM:HAM's F12 medium. The outer compartment of the Transwells was filled with 1.5 ml DMEM:HAM's F12 medium containing 10 % FCS and 10 % normal human serum (NHS) as well as penicillin/streptomycin. Radioactivity in pooled culture medium (inner + outer compartment) and cartilage-like slices was determined after 7 days of co-culture (without intermediate refreshment of the culture medium). Cartilage-like slices were digested in 0.5 ml Soluene (Amersham) for 3 hours in a 50 ºC shaking incubator. This digest was added to 9.5 ml of UltimaGold scintillation fluid (Amersham) and radioactivity was measured by a liquid scintillation analyzer (Tri-Carb 1900 CA, Packard). The same was done for 0.5 ml of medium. The percentage of matrix degradation was calculated as: $(dpm_{[medium]}/(dpm_{[medium]} + dpm_{[matrix]}))*100\%$.

Statistical analysis

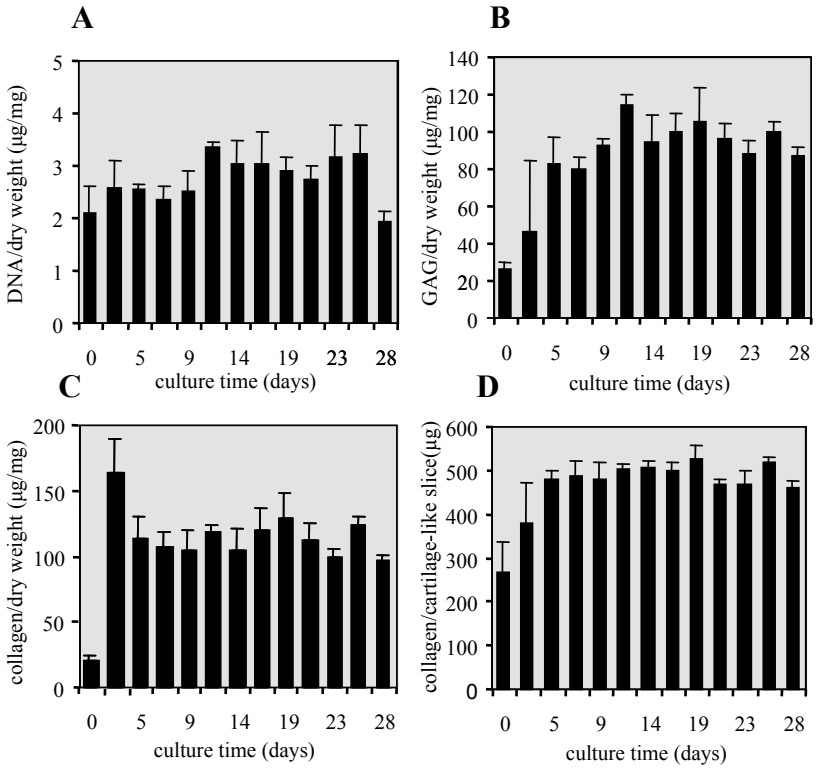
For statistical analysis, means and standard deviations were calculated. Differences between conditions were tested for statistical significance using the Kruskall-Wallis test with a *post hoc* Mann-Whitney U-test. Differences were considered statistically significant at $p<0.05$. All statistical analyses were performed using SPSS 11.5 Software (SPSS, Chicago, IL).

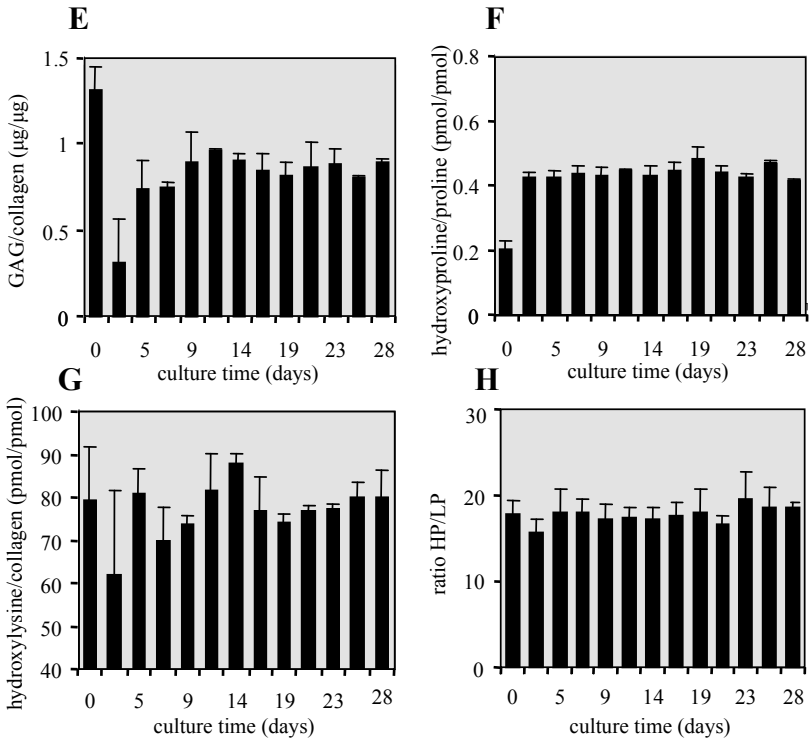
Results

Biochemical analysis of cartilage-like slices

Cartilage-like slices were analysed biochemically to study the resemblance to native cartilage. Some known differences with native cartilage were observed, probably due to relatively high cell numbers and relatively short culture period. As expected, the cellularity of the matrix was higher in cultured slices compared to the amount in native cartilage (2.74 ± 0.24 µg DNA/mg tissue after three weeks of culture and 0.24 ± 0.05 respectively) (figure 1A). To determine the amount of extracellular matrix that was formed, the levels of proteoglycans (assessed as GAG) and collagens (assessed as hydroxyproline) were measured. The amount of GAG/dry weight increased from 26.6 ± 3.6 µg /mg immediately after transfer to Transwells to around 90-100 µg /mg after nine

Figure 1. *Extracellular matrix content of in vitro engineered cartilage-like slices. Slices were cultured for 28 days, every 2 days 3 slices were frozen for biochemical analysis. Time point 0 is the cell suspension after dissolving of the alginate beads. DNA per dry weight (**A**) was taken as a measure for the amount of cells present. As measures for the amount of matrix produced, GAG/dry weight (**B**), collagen/dry weight (**C**) and collagen per cartilage-like slice (**D**) were determined. In addition, as measures for the quality of the produced matrix, GAG/collagen (**E**), hydroxyproline/proline (**F**), hydroxylysine/collagen (**G**), and ratio of HP and LP crosslinking were determined.*





days of culture (figure 1B). After these nine days in culture, the amount of GAGs was relatively stable, but still lower then in normal articular cartilage (145.4 ± 96.4). In addition, normal bovine articular cartilage contained more collagen per dry weight (550 ± 163 µg/mg) then the slices in culture (112 µg/mg) (figure 1C). The amount of collagen per total dry weight increased in the first week after the transfer to Transwells and remained relatively stable from thereon (figure 1D).

As a measure for the quality of cartilage often the ratio of GAGs per collagen is determined. The level of GAG/collagen in normal bovine articular cartilage was 0.3 (± 0.08) µg/µg. After an initial drop in GAG/collagen content when chondrons were transferred from alginate to the Transwells system, an increase in GAG/collagen was observed (figure 1E). After 7-10 days in Transwell culture, the level of GAG/collagen remained relatively stable at approximately 0.9 µg/µg (three times higher than in native cartilage). As another measure for the amount of collagen per total protein, the amount of hydroxyproline (a collagen-specific amino acid) was compared to the amount of proline (which is present in all proteins). The ratio of hydroxyproline over proline is 0.72 (± 0.16) pmol/pmol in native articular cartilage, while in our *in vitro* engineered cartilage-like

slices this ratio was 0.6 times lower. After two days of culture in Transwells the ratio was 0.43 (± 0.01) and remained stable thereafter (figure 1F).

***Table 1**. Biochemical analysis of native bovine cartilage compared to in vitro engineered cartilage-like slices after three weeks of culture in Transwells, the time at which they are used for coculture and stimulation experiments.*

	native bovine cartilage	cartilage like slices:	statistics (p value)
DNA/dry weight (µg/mg)	0.24 ± 0.05	2.74 ± 0.24	0.036
GAG/dry weight (µg/mg)	145.4 ± 24.9	96.4 ± 8.3	0.036
collagen/dry weight (µg/mg)	549.1 ± 162.7	112.5 ± 12.8	0.036
GAG/collagen (µg/µg)	0.28 ± 0.08	0.87 ± 0.15	0.036
hydroxyproline/proline (pmol/pmol)	0.72 ± 0.16	0.44 ± 0.02	0.036
hydroxylysine/collagen (pmol/pmol)	62.9 ± 6.2	77.0 ± 1.2	0.036
ratio HP/LP	59.1 ± 5.4	16.7 ± 1.0	0.036

Two measures for the proper posttranslational modification of the newly formed collagen are the level of hydroxylation (hydroxylysine/collagen) and the ratio of HP over LP crosslinking. The amount of collagen modification is an indication for the quality of collagen which is formed. In our cartilage-like slices we found a lower ratio of HP/LP crosslinking than found in normal bovine articular cartilage (around 17-18 and 59 ± 5.4 respectively) (figure 1H). The ratio of crosslinking in the cartilage-like slices was still increasing however. The number of hydroxylysine residues per collagen was 63 (± 6.2) pmol/pmol collagen in normal articular cartilage, in the cultured cartilage-like slices it was around 77 pmol/pmol collagen (figure 1G). This indicates that the lysyl hydroxylation of the collagen type II resembles that of normal bovine articular cartilage.

Coculture of cartilage like slices with RA synoviocytes

To study the interaction between chondrocytes and synoviocytes, RA synoviocytes were cultured on top of the cartilage-like slices for 7 days. After 7 days of coculture without medium refreshment, an increased ^{35}S release is observed when RA synoviocytes were

cocultured with living chondrocytes (123 ± 27 %; n=7) (*P*=0.035), indicating an increased cartilage matrix degradation by chondrocytes, synoviocytes, or both (figure 2). To investigate whether the increased cartilage release was caused directly by active synoviocytes or resulted from the interaction between synoviocytes and chondrocytes, synoviocytes were seeded on cartilage in which chondrocytes were freeze-killed. After culture of synoviocytes on cartilage with killed chondrocytes, no increase in ^{35}S release was seen (97 ± 18 %; n=7) (*P*=NS), indicating that an interaction between synoviocytes and living chondrocytes is essential for the synoviocyte induced cartilage degradation of live cartilage.

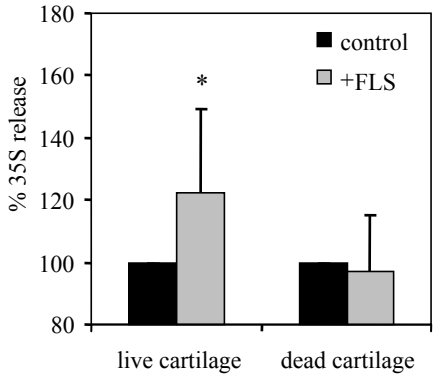


Figure 2. *^{35}S release of cartilage-like slices after interaction between chondrocytes and synoviocytes.*

In addition, the effect of IL-1β and TNF-α, both macrophage-derived cytokines, was studied. Stimulation of living cartilage-like slices with IL-1β and TNF-α resulted in an upregulation of ^{35}S release of 250 ± 110% (n=7) (*P*=0.004) and 190 ± 64 % (n=7) (*P*=0.004) respectively (figure 3). No additional effect of synoviocytes was seen (238 % ± 99 and 200 % ± 75 respectively) (figure 4). This absence of induction by synoviocytes could be caused by two factors: there are 40-50 times more chondrocytes than synoviocytes in each culture, so induction of degradation by synoviocytes could be lost in the response by the chondrocytes or the amount of IL-1α and TNF-α added to the culture has reached a threshold level so the cytokines produced by the synoviocytes do not have an additional effect.

When synoviocytes were seeded on cartilage with freeze killed chondrocytes, also no increase of ^{35}S release was seen. Addition of IL-1β and TNF-α to the culture of synoviocytes on dead cartilage also had no effect on ^{35}S release (figure 4).

Together these results show an interplay between synoviocytes and chondrocytes in the breakdown of cartilage, as well as a strong induction of chondrocyte mediated cartilage-breakdown by the proinflammatory cytokines IL-1β and TNF-α.

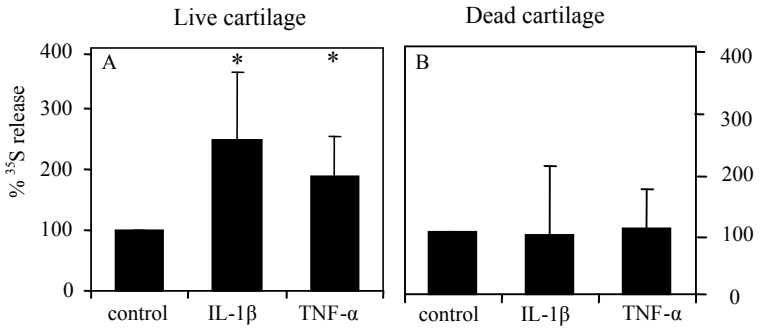


Figure 3. *^{35}S release after stimulation of live cartilage-like slices **A)** and dead cartilage-like slices **B)** with IL-1β or TNF-α.*

Discussion

In the present study we describe the use of the alginate recovered chondrocyte method in a novel, medium-throughput model, employing human synoviocytes and bovine chondrocytes. In view of the relevance for human pathology, the use of human cells is preferred over animal cells. However, adult human articular cartilage is scarcely available. Bovine articular chondrocytes have a virtually unlimited availability and are widely accepted and used as a substitute for human articular chondrocytes in *in vitro* studies (14-16).

Therefore, in this model we combined human fibroblasts with bovine articular chondrocytes, making it possible to test many different conditions of cartilage destruction. Our *in vitro* engineered cartilage resembled native bovine cartilage quite well, although also in our system the amount of collagen was relatively low, a known problem in cartilage tissue engineering. The ratio of HP/LP crosslinking was lower in our cultured cartilage, resembling the level of crosslinking ratio in adult human cartilage rather than young bovine cartilage (unpublished observation).

The major benefits of our *in vitro* engineered cartilage over existing *in vitro* models are the composition of the matrix, which resembles real cartilage with its most abundant components collagen type II and proteoglycans, and the presence of chondrocytes instead of a collagen type I or type IV matrix without cells. One of the reasons collagen type II is preferred is because while several MMPs are able to degrade the collagen type IV present in Matrigel, the most important MMPs in the degradation of cartilage are the collagenases MMP-1 and MMP-13. MMP-1 and -13 are the only MMPs able to initiate the degradation of collagen type II (17). MMP-1 can be produced by both synoviocytes and chondrocytes while MMP-13 is characteristically produced by activated chondrocytes (17-20). In

addition, breakdown of proteoglycans might be a crucial step in cartilage degradation; *in vivo* data have shown that active ADAMTS-5, an enzyme involved in proteoglycan degradation, is essential for cartilage degradation in an osteoarthritis mouse model (21). This again emphasises the importance to include all components of native cartilage.
Previously, Pap et al (11) showed that storage of cartilage, or inhibition of chondrocyte protein synthesis reduced degradation of cartilage matrix in the presence of synoviocytes. Our study confirms these data by showing that synoviocytes are unable to degrade the *in vitro* engineered cartilage in the absence of live chondrocytes. The drawback of using explants of cartilage-slices is that the cartilage often has to be stored, which influences the reaction of chondrocytes. In addition, because the matrix synthesis rate in our engineered cartilage is greater than in cartilage explants, the amount of radioactive tracer molecules incorporated into the proteoglycans of the matrix is higher. Therefore the sensitivity in our model will be greater than in models using cartilage explants.

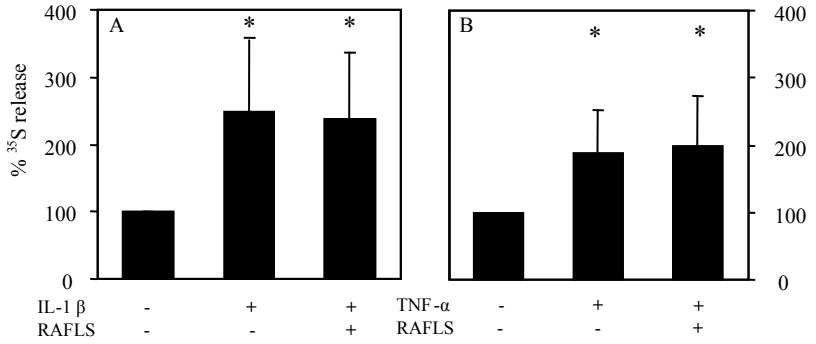


Figure 4. *^{35}S release of cartilage-like slices during synoviocyte -chondrocyte cocultures after stimulation with IL-1β* ***A)*** *or TNF-α* ***B)****.*

IL-1β is an important mediator of RA and a known stimulator of both chondrocytes and synoviocytes (10;22-25). In chondrocytes it upregulates production of MMP-1 and MMP-13 (25) and in synoviocytes, IL-1β induces production of inflammatory cytokines and pro-MMP-1 and also stimulates proliferation (26). IL-1β is mainly produced by monocytes/macrophages, but it is also suggested that IL-1β plays a role in the interaction between synoviocytes and chondrocytes (11). Neidhart *et al* found that inhibition of IL-1β in a coculture of chondrocytes with synoviocytes inhibited the degradation induced by synoviocytes, also indicating a role for IL-1β in the interaction between chondrocytes and synoviocytes (10). Although we found an effect of IL-1β on chondrocytes, we did not find an additional effect on synoviocytes. This could be the result of the difference in cell numbers (around $6x10^6$ chondrocytes versus $1x10^5$ synoviocytes in our model) or because

of the relatively high concentration of IL-1β added to the culture. Possibly, chondrocytes are already maximally activated by IL-1β so addition of synoviocytes cannot induce any increase in cartilage degradation.

Similar to IL-1β, TNF-α plays an important role in RA pathology. While IL-1β is thought to play a role in later stages of disease, TNF-α is predominantly detected at early stages of disease (2). TNF-α plays an important role in the invasive behaviour, proliferation, and cytokine production of synoviocytes. In addition, it also downregulates matrix production and upregulates cytokine and MMP production in chondrocytes (27;27-30). In our model, TNF-α is able to induce cartilage degradation comparable to IL-1β. Again no additional stimulation of cartilage degradation was observed in the coculture setup, suggesting TNF-α might play a role in our coculture system similar to that of IL-1β and similar to its role in RA pathology. This indicates TNF-α can be one of the cytokines through which synoviocytes and chondrocytes communicate with each other and with macrophages. Since macrophages are the main producers of both IL-1β and TNF-α, the role of macrophages in cartilage degradation should not be neglected. Therefore it seems that in models like the one described in this paper, inclusion of macrophages or addition of cytokines produced by activated macrophages may lead to a further optimalisation of the model.

Although the model described in our study was designed to study the direct interaction of synoviocytes and chondrocytes in RA, it can easily be adjusted to study cartilage degradation in osteoarthritis. In the current view, in RA, the contact of the pannus tissue with the articular cartilage is responsible for the invasion and degradation of the articular cartilage (18). In contrast, it is thought that in osteoarthritis, chondrocytes are primarily responsible for cartilage degradation (31). A hyperplasive synovium is however also found in patients with osteoarthritis, therefore the interaction between chondrocytes and synoviocytes might also play a role in osteoarthritis, but maybe by soluble factors in stead of by cell-cell contact (32). In concordance with such view, synoviocytes could be separated from the chondrocytes in our model by seeding them in the inner compartment of the Transwell. By that manner, interaction via soluble factors remains possible, while cell-cell contact is absent.

In conclusion, the use of the alginate-recovered-chondrocyte method provides a novel model for cartilage degradation. Comparable to findings in other *in vitro* models with real cartilage and *in vivo*, in this model, the role for synoviocytes in cartilage degradation in RA seems to be dependent on the presence of living chondrocytes. Moreover, addition of proinflammatory cytokines stimulated cartilage-breakdown only when living chondrocytes were present, but not in the presence of living synoviocytes. Therefore, the effect of cytokines on this interaction seems to be mostly directed on chondrocytes. This indicates that in using models to study cartilage degradation, it is crucial to use a matrix resembling real cartilage with living chondrocytes and to include more than one cell type,

especially since our data suggest that synoviocytes might not be able to degrade cartilage themselves.

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Chapter 3

Activation of receptor for advanced glycation end products in osteoarthritis leads to increased stimulation of chondrocytes and synoviocytes

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Abstract

Objective: *The major risk factor for osteoarthritis (OA) is aging, but the mechanisms underlying this risk are only partly understood. Age-related accumulation of advanced glycation end products (AGEs) could be one of these mechanisms. Here we investigate the role for the receptor for AGEs (RAGE), in mediating the cellular effects of AGEs on chondrocytes and fibroblast-like synoviocytes (FLS).*

Methods: *AGE levels in human cartilage were determined by fluorescence, browning and pentosidine levels. Chondrocyte activation by AGEs was assessed as the release of proteoglycans and the synthesis of matrix metalloproteinase 1(MMP-1) and type II collagen messenger RNA (mRNA). The activation of FLS by AGEs was measured by MMP-1 production and invasion through matrix proteins.*

Results: *Patients with focal degeneration of cartilage showed increased AGE levels in their healthy cartilage compared with levels in healthy cartilage from donors without cartilage degeneration ($p<0.0$ for both fluorescence and browning; p not significant for pentosidine content). Stimulation of bovine chondrocytes with glycated albumin increased the release of proteoglycans by 110% ($p<0.001$) and the production of MMP-1 mRNA by 200% ($p=0.028$). In addition, OA FLS produced 240% more MMP-1 when stimulated with glycated albumin ($p<0.001$). Glycated matrix or albumin stimulated the catabolic activity of OA-FLS, which was assessed as invasive behaviour, by 150% and 140% respectively ($p=0.001$ and $p=0.01$). Effects of stimulation with AGEs were blocked by a neutralizing antibody against RAGE, but not by an isotype control.*

Conclusion: *This study shows that AGEs trigger RAGE on chondrocytes and FLS, leading to increased catabolic activity and therefore cartilage degradation. AGEs, via RAGE, could therefore contribute to the development and/or progression of OA.*

Introduction:

Osteoarthritis (OA) is one of the most common forms of arthritis (1;2). OA is a progressive degenerative joint disease which has a major impact on joint function and the patients' quality of life. Many risk factors that contribute to disease onset have been identified, including systemic factors, such as genetics, estrogen use, and bone density and local biomechanical factors, such as muscle weakness, obesity, and joint laxity (1). The most important risk factor for OA besides female sex, obesity, and joint trauma is aging (1-3). However, it is relatively unknown how aging contributes to the onset and progression of OA.

A prominent feature of aging is the modification of proteins by non-enzymatic glycation. Non-enzymatic glycation is a common posttranslational modification of proteins caused by reducing sugars. The spontaneous condensation of reducing sugars with free amino groups in lysine or arginine residues on proteins, leads to the formation of a reversible Schiff-base, which is subsequently stabilized by Amadori rearrangement. Next, Maillard

or browning reactions convert the initially formed intermediate products into advanced glycation end products (AGEs) (4).

In addition to this classical pathway of AGE formation, it has recently been found that AGE formation can be initiated by metal-catalyzed glucose auto-oxidation, as well as by lipid peroxidation (thereby providing an interesting link between lipid metabolism and development of OA). This diversity in reaction pathways results in a variety of chemical structures of AGEs. Some AGEs are adducts to proteins, while many others present protein-protein crosslinks. Once they are formed, AGEs cannot be removed from the protein and therefore, AGEs only leave a tissue when the protein involved is degraded. Articular cartilage collagen has an exceptionally long half-life, and since the rate of AGE accumulation is largely determined by the rate of protein turnover (5), this low turn over results in an abundant accumulation of AGEs in articular cartilage (6;7). The accumulation of AGEs in cartilage leads to inferior mechanical properties (6;8) and an alteration in cartilage metabolism (5;9). More specifically, cartilage stiffness increases substantially with increasing AGE levels, and matrix synthesis by articular chondrocytes becomes impaired (6;8;10). *In vivo* effects of AGEs have been identified in a recent study in a canine model of OA induced experimentally by anterior cruciate ligament transection. Animals with elevated AGE levels had more severe OA than did those with normal AGE levels (11).

The mechanism by which AGEs influence cellular function in articular cartilage is poorly understood. The alteration in matrix synthesis might be mediated by receptors for AGEs. Several of such AGE receptors have been identified, scavenger receptors type I and II, oligo saccharyl transferase 48 (AGE-R1), 80 K-H phosphoprotein (AGE-R2), galectin-3 (AGE-R3) and the receptor for advanced glycation end products (RAGE) (4). RAGE is a multiligand member of the immunoglobulin superfamily of cell surface molecules, and is present on a diversity of cell-types (12-14). RAGE and accumulation of its ligands, namely AGEs, amyloid fibrils, S100/calgranulins, and high mobility group box -1 (previously called amphoterin), are implicated in several pathologic conditions, such as diabetes, immune/inflammatory disorders, dialysis-related amyloidosis, and tumors (15-17). In some of these diseases, RAGE activation by its ligands leads to an increased inflammatory response, but in other diseases, it also plays a role in cell migration and/or tumor metastasis (14;15;18;19).

Although synovial inflammation is usually mild and seems to play a secondary role in OA, fibroblast-like synoviocytes (FLS) may very well be involved in OA pathology. (20) A role for RAGE in the activation of FLS has been suggested in studies focussing on inflammatory arthritis but has not been studied for OA. Both the receptor (RAGE) and its ligand (the AGEs) are present in the synovial lining, sublining, and endothelium of OA synovial specimens (21;22). In synovium, RAGE is present on a number of different cell-types, including FLS. Levels of AGEs are increased in serum, synovial fluid, and urine

from OA patients as compared with controls (21;23).It was found that β2-microglobulin (β_2M) modified by AGEs induced the release of monocyte chemo-attractant protein-1 (MCP-1) by FLS and the production of collagen type I by skin fibroblasts respectively, suggesting a stimulatory effect of RAGE activation on fibroblasts (16;24). Blockade of RAGE in mice immunized and challenged with collagen type II (CII) inhibited not only inflammation, but also cartilage degradation (12), a finding that supports the notion of a role for RAGE in joint diseases. In OA, RAGE activation might therefore not only play a role in the initiation of the disease (altered matrix synthesis by chondrocytes and altered biomechanical properties), but it might also influence progression of the disease by activating FLS.

The purpose of the current study was to test the hypothesis that AGEs, via binding to RAGE, may contribute to the propagation of tissue destruction in OA. To corroborate existing data that AGE accumulation predisposes to the development of OA, we measured the level of AGEs in normal-appearing cartilage obtained from healthy subjects and from patients with focal cartilage degeneration. Next, we studied the effects of these AGEs on chondrocyte and synovial fibroblast activation via RAGE. Activation of chondrocytes was tested by their ability to degrade their own matrix and by studying production of matrix metalloproteinase 1 (MMP-1) and CII. The activation of catabolic processes of FLS was studied using an *in vitro* invasion assay and by measuring production of active and proMMP-1.

Material and methods

Cartilage samples for biochemistry. Cartilage specimens obtained at autopsy within 18 hr after dead were used for the analysis of the biochemical composition of the extracellular matrix (AGE levels, $_{D}$-Asp [the racemized form of $_{L}$-Asp], and collagen). These parameters are not influenced by the time between death and cartilage harvesting. Donors had no known clinical history of joint disorders. Donors were selected either for knees with focal degenerative cartilage (20) or for knees without any sign of degeneration (22). Macroscopically normal cartilage from knees with focal cartilage degeneration (N_{DEG}; Mankin grade 0-1) (25) was compared to cartilage from knees without any sign of degeneration (control; Mankin grade 0-1) (26). Cartilage from a total of 22 donors with focal cartilage degeneration and 23 age-matched controls was used. The ages of the donors ranged from 46 to 85 years (mean age ± SEM 69.5 ± 2.0 years in the macroscopically normal donor group and 66.3 ± 2.5 years in the control group; P not significant [NS]). For each donor, we analyzed 3 cartilage samples that were taken randomly from the weight-bearing areas of the condyles of both knees. It was demonstrated that the mean value of 3 random samples was representative of the entire joint for all parameters tested (data not shown). All tissue samples were stored at -20°C until analyzed.

Cartilage samples for reverse transcriptase polymerase chain reaction (RT-PCR). Human articular cartilage was collected from 6 OA donors undergoing total knee replacement surgery and from 2 normal donors postmortem. The cartilage was snap-frozen in liquid nitrogen and stored at -80 ºC until further processing.
Cartilage was milled into a fine powder and total RNA was extracted and purified from the powder using the Qiagen RNeasy midi kit (Qiagen, Hilden, Germany) with an on-column DNase digestion. Purity of the isolated RNA was determined by measuring optical density at 260/280 nm using a ND-100 spectophotometer 9Nanodrop Technologies, Wilmington, DE). The amount of RNA purified was determined using the RiboGreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). The messenger RNA (mRNA) fraction of the total RNA was converted to complementary DNA (cDNA) using a first strand cDNA synthesis kit for RT-PCR (Roche Diagnostics, Mannheim, Germany) and oligo (dT) primers.

Purification of collagen from cartilage. Articular cartilage collagen was purified by depleting the tissue of all proteoglycans and other non-collagenous proteins by sequential enzymatic treatment with chondroitinase ABC (Sigma, St. Louis, MO), trypsin (Boehringer, Mannheim Germany) and S*treptomyces* hyaluronidase (Sigma) at 37 ºC as described previously (5).

Analytical procedures. Collagen-linked fluorescence excitation and emission spectra (λ_{ex}=360 nm, λ_{em}=460 nm) and browning (absorption at 340 nm) were measured in papain digests of cartilage collagen (0.5-2 mg collagen in 250 µl of papain buffer) as described elsewhere (11). One aliquot of the papain digests (100 µl) was hydrolyzed in 1 ml of 6 M HCl at 110°C for 20-24 h for cross-link and amino acid analysis, while another aliquot (25 µl) was hydrolyzed in 1 ml of 6 M HCl at 100°C for only 4 h for % $_{D}$-Asp analysis, as described below. Both fluorescence and browning were expressed in relative units and normalized to the Hyp content of the digest that was obtained from amino acid analysis (papain contributed <1% of the Hyp in the digests).
Collagen pentosidine content and amino acid composition were determined by high-performance liquid chromatography (HPLC). After hydrolysis (20-24 hours) and subsequent drying, papain digests were dissolved in water containing the internal standards pyridoxine (10 µM; Sigma) and homoarginine (2.4 mM; Sigma). For collagen pentosidine analysis, samples were diluted five-fold with 0.5% (volume/volume) heptafluorobutyric acid (Fluka, Buchs, Switzerland) in 10% (v/v) acetonitrile (Rathburn, Walkerburn, UK) and analyzed by HPLC (27). For amino acid analysis, an aliquot of the cross-link samples was diluted 20-fold with 0.1 M borate buffer (pH 11.4), derivatized with 9-fluorenylmethyl chloroformate (Fluka) and analyzed by HPLC (28). The

pentosidine content of the collagen samples is expressed as mol per mol collagen, assuming 300 hydroxyproline (Hyp) residues per triple-helical collagen molecule (27).
The percentage $_{D}$-Asp (% $_{D}$-Asp) in cartilage collagen was determined by HPLC in aliquots of the papain digests that had been briefly (4 h) hydrolyzed in 6 M HCl (5). Dried hydrolyzates were dissolved in 1 ml of 0.1 M sodium borate buffer (pH 9.5). An aliquot of the samples (20 µl) was derivatized with 5 µl reagent containing *o*-phtaldialdehyde (30 mM; Sigma) and *N*-acetyl-L-cysteine (60 mM; Sigma) in 30% (v/v) methanol (Rathburn) in 0.1 M sodium borate buffer (pH 9.5) and analyzed by HPLC (5). All of % $_{D}$-Asp data were corrected for the amount of $_{D}$- and $_{L}$-Asp present in papain (10-20% of the aspartic acid in the samples) and for racemization during the hydrolysis step.

Chondrocyte isolation, culture, and stimulation. Chondrocytes were isolated from the metacarpophalangeal joints of 6-month-old fat calves. Cartilage was harvested and digested with 0.2% pronase (Merck, Darmstadt, Germany) for 1 hour, followed with overnight digestion with 0.025% collagenase (CLS2, Worthington, Freehold, NJ). Cells were separated from the undigested tissue using a 200 µm filter (B-Braun Medical, Oss, the Netherlands) and cultured in 1.2% low viscosity alginate (Keltone, Monsanto Pharmaceutical Ingredients, Chicago, IL) at 4 x 10^6/ml for 14 days in DMEM (GibcoBRL, Paisley, UK), supplemented with 0.28 mM ascorbic acid (Sigma-Aldrich, Steinheim, Germany), 100 IU/ml penicillin (BioWhittaker, Versiers, Belgium), 100 µg/ml streptomycin (BioWhittaker) and 10% foetal calf serum (FCS, GibcoBRL) as described previously (29).
To induce glycation, bovine serum albumin (BSA; Sigma) was incubated in the presence of 10 mM $_{D}$-ribose (Sigma) for 3 days in DMEM:HAM's F12 at 37ºC, after which it was extensively dialyzed to remove excess ribose. Chondrocytes were cultured as described above for three weeks, after which they were washed three times for one hour with serum-free DMEM. Beads were then placed in a 24 well plate) (Costar, Cambridge, MA) at a density of 10 per well in 0.5 ml medium. Chondrocytes were stimulated for 48 hours with BSA or glycated BSA (BSA-AGE); beads and medium were stored separately at -20ºC until analyzed.

Proteoglycan measurement. Using a commercial kit (Biocolor Ltd, Belfast, N. Ireland), the amount of sulfated glycosaminoglycans (GAGs), reflecting the amount of proteoglycans, in culture medium or alginate beads was determined. Prior to measurement, beads were digested for 24 hr at 56ºC in papain buffer containing 3% v/v papain, 5 mM cysteine HCl, 50 mM EDTA, and 0.1 M sodium acetate (pH 5.53). Culture media were measured without papain digestion. Proteoglycan degradation was expressed as the percentage of GAG released into the medium.

Real-time PCR on bovine chondrocytes. Bovine chondrocytes were stimulated as described above. Synovial fibroblasts were seeded at a density of 100.000 cells per well in a 6 well flat-bottom plate 2 days prior to RNA isolation. RNA was isolated using the Qiagen RNeasy mini kit and subsequently converted to cDNA using avian myeloblastosis virus reverse transcriptase (Roche Diagnostics) and subjected to real-time PCR amplification. Real-time PCR amplification was performed for MMP-1, CII) and the housekeeping gene glyceraldehydes GAPDH. Complementary DNA was amplified using specific primers and specific beacons (table I and II respectively) in a total reaction volume of 25 µl containing 1x PCR buffer (Applied Biosystems), 0.4 mM of each dNTP, 2.5 mM $MgCl^{2+}$, 500 nM of each primer and beacon, and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems). PCR was performed using an ABI PRISM 7700 sequence detection system and consisted of a 5-minute interval at 95 ºC followed by 40 cycles of 95 ºC for 30s, 56 ºC for 40s, and 72 ºC for 30s. Data were analysed using Sequence Detector version 1.7 software (Applied Biosystems).

***Table 1**. Primers for real-time polymerase chain reaction **

Name	Forward primer	Reverse primer
bovine GAPDH	5' CCCTGAGCTCAACGGGAAGC 3'	5' GTTTCTCCAGGCGGCAGGTC 3'
bovine MMP-1	5' ACCCCAGACCTGTCAAGAGCA 3'	5'TCCCCTCACAAAGGATATCATTATG 3'
bovine collagen II	5'AAGGTTTTCTGCAACATGGAGACT 3'	5'AGATGTGTTTCTTCTCCTTGCTCTT 3'
human RAGE	5'-CAGTAGCTCCTGGTGGAACCGTAAC-3'	5'- CCTATCTCAGGGAGGATCAGCACAG-3'
human β_2M	5'TCTTGTACTACACTGAATTCACCCCC ACTGA 3'	5'ATCCAAATGCGGCATCTTCAAACCTC3'

** MMP-1 – Matrix Metalloproteinase 1; RAGE – receptor for advanced glycation end products; β2M – β2-microglobulin.*

***Table 2.** Molecular beacons for real-time polymerase chain reaction**

Name	Sequence †
bovine GAPDH	5' FAM ctgccGCCTTCCGCGTCCCCACTCCggcag-DABCYL 3'
bovine MMP-1	5' FAM-cgtgccAAAGCCTTTCAACTCTGGAGCAATGTCACggcacg-DABCYL 3'
bovine collagen II	5' FAM-cgtgcGTCTACCCCAACCCGGCCAGCgcacg-DABCYL 3'
human RAGE	5' FAM-cgtgccGCCCAGCCCTCTCCTCAAATCCAggcacg- DABCYL 3'
human β_2M	5'FAM- cgtgcCCTGCCGTGTGAACCATGTGACTTTGgcacg – DABCYL 3'

**FAM – 6-carboxyfluorescine; DABCYL – 4-(4-dimethylaminophenyl)benzoid acid, see Table 1 for further definitions.*

† Lower case letters represent base pairs added for the formation of a hairpin loop structure of the beacon, quenching its signal when it is not bound to the DNA.

Real time PCR on FLS and human articular cartilage. RAGE and the housekeeping gene β_2M were amplified using specific primers and specific beacons (tables I and II, respectively) in a total reaction volume of 25 µl containing 1x PCR buffer (Applied Biosystems), 0.4 nM of each dNTP, 3.5 mM Mg^{2+}, 500 nM of each primer, and 1 unit of AmpliTag Gold polymerase (Applied Biosystems). PCR was performed in an ABI PRISM® 7700 sequence detection system and consisted of a 5-minute interval at 95 ºC followed by 40 cycles of 95 ºC for 30s, 56 ºC for 40s, and 72 ºC for 30s. Data were analyzed using Sequence Detector version 1.7 software.
For detection of RAGE product on gel, a PCR of a 5-minute interval at 95 ºC followed by 35 cycles of 95 ºC for 30s, 56 ºC for 40s, and 72 ºC for 30s in a model 9600 thermal cycler (PerkinElmer Life Sciences, Wellesly, MA) was performed using the same reaction mixture as described above. PCR subjected to gel electrophoresis using a 2% agarose gel containing ethidium bromide to visualize the amplified fragment.

Culture of synovial fibroblasts. Synovial fibroblasts were derived from tissues obtained at joint replacement surgery or synovectomy from subjects who had given their informed consent. Tissue was harvested by an orthopedic surgeon and collected in sterile phosphate buffered saline (PBS). Fat and connective tissue were removed, and tissue was digested with collagenase for at least 2 hours at 37°C. Cells were then separated from the undigested tissue using a 200 µm filter (B-Braun Medical) and cultured in 162-cm^2 culture flasks (Costar) with Iscove's Modified Dulbecco's medium (IMDM; BioWhittaker) supplemented with glutamax (BioWhittaker), 100 U/ml penicillin and streptomycin (BioWhittaker), and 10% FCS at 37°C in a humidified atmosphere of 5% CO_2 in air. Upon reaching confluence, cells were detached with 0.25% trypsin and split in a 1:3 ratio. For all experiments third-passage synovial fibroblasts were used. Using light microscopy 99% of cells were judged to be FLS.

Invasiveness of FLS in Matrigel matrix. Invasion assays were performed using a transwell invasion system with Matrigel (30). Prior to coating with Matrigel (Matrigel basement membrane matrix, Becton Dickinson, Bedford, MA), the sides of the transwells (with 6.5 mm diameter polycarbonate filters with 8.0µm pore size; Costar) were coated with paraffin to prevent meniscus formation. Transwells were then incubated with IMDM for 30 minutes at 37 °C. Matrigel was diluted in serum-free IMDM to a concentration of 0.375 mg/ml. After removal of the medium, transwells were coated with 100 µl of 0.375 mg/ml Matrigel in IMDM overnight in a full functional laminar flow cabinet. For AGE experiments, transwells were coated with Matrigel as described above. After drying overnight, the Matrigel was incubated in the presence or absence (control) of 200 mM $_D$-ribose (to induce AGEs) (Sigma) in IMDM. After three days, the ribose solution was removed and the Matrigel was washed with IMDM to remove the remaining free ribose.

The next day the Matrigel-coated transwells were pre-incubated with 100 µl IMDM for 1 hour at 37°C in a humidified atmosphere of 5% CO_2 in air. Medium was removed, and 20,000 synovial fibroblasts in serum-free IMDM were seeded on the Matrigel transwells. The lower compartment was filled with 0.9 ml IMDM containing 10% FCS and 10% human serum. The serum-free medium in the inner compartment was refreshed daily when antibody was added. After three days, the cells were fixed with 4% formaldehyde for 30 minutes at room temperature. After removal of the formaldehyde, cells were washed with demineralized water and stained with 1% crystal violet in PBS for another 30 minutes at room temperature. The cells were again washed with water and the Matrigel and noninvading cells were removed by cleaning the inner compartment with a cotton bud. The number of cells that had invaded the Matrigel and grown through the transwell membrane was counted using a light microscope. Experiments were all performed in triplicate (31).

MMP-1 production. Bovine serum albumin (Sigma) was dissolved in 4 mg/ml in IMDM and was glycated by addition of 10 mM $_D$-ribose for 3 days at 37ºC. After this period, the glycated BSA was extensively dialysed to remove excessive ribose. FLS were plated at a density of $0.2*10^6$ cells per well in a 12 well flat-bottomed culture plate (Costar) 1 day prior to stimulation (n=3 wells per condition) and were placed in serum-free medium containing 0.1% lactalbumin hydrolysate 1 hour before stimulation. Cells were incubated for 48 hours in medium with 0.1% lactalbumin, and 4 mg/ml glycated BSA, or 25 ng/ml TNF-α. To block RAGE activation, 2.5 ng/ml anti-RAGE antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added. At the end of the incubation time, supernatants were collected from the culture, aliquoted, and frozen at -80ºC until analysis was performed.

Measurement of human MMP-1 by sandwich enzyme linked immunosorbent assay (ELISA). This assay is based on a 2-site ELISA sandwich format and was carried out according to the manufacturer's instructions (Biotrak™, Amersham-Pharmacia Biotech, Piscataway, NJ). Plates were coated overnight at 4˚C with anti MMP-1 antibody, which recognizes total human MMP-1 (both pro- and processed form). The next day, plates were incubated for 2-4 hours with Stabilcoat (Surmodics, Eden Prairie, MN) at 25˚C, after which they were washed four times with PBS-Tween and incubated with 5 x diluted culture supernatant or a standard curve of MMP-1. The medium was allowed to bind overnight at 4˚C. Units of active and proMMP-1 were determined by measuring the resulting colour at 405 nm in a microtiter plate spectrophotometer after activation of all proMMP by APMA and addition of detection reagent, containing 2 substrates for MMP-1.

Statistical analysis. Statistical analysis was performed using SPSS 11.0 software (SPSS, Chicago, IL). Multiple comparisons were performed by analysis of variance or Friedman's test, and individual differences were tested by *post hoc* Bonferroni test or Mann-Whitney-U tests. In figure 1 data is represented as mean ± SEM; in all other figures, data are expressed as mean ± SD.

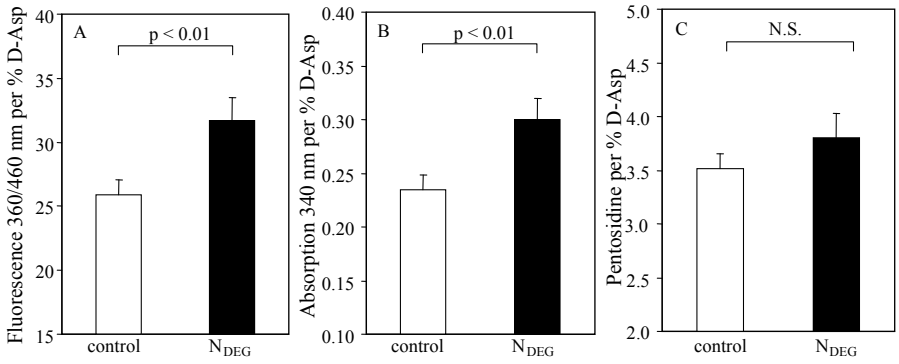

Figure 1. *Levels of advanced glycation end products (AGEs) crosslinks measured as fluorescence, browning and pentosidine content. AGE levels were corrected for the collagen residence time (% $_D$-Asp [the racemized form of $_L$-Asp]) in collagen from control cartilage (n = 22) and macroscopically normal cartilage from knees with focal degeneration (N_{DEG}; n = 23) (see Materials and Methods).* ***A****, fluorescence (λ_{ex} = 360 nm, λ_{em} = 460 nm) per nmol of Hyp.* ***B,*** *Browning (absorption 340 nm per µmol Hyp).* ***C****, Pentosidine content (mmoles per mole of collagen). Corrected AGE levels, i.e. the rate of AGE formation, are higher in collagen from N_{DEG} cartilage than in control cartilage collagen. Values are the mean and SEM. NS = not significant.*

Results

AGE levels in cartilage. In order to corroborate the importance of AGE accumulation in the development of OA, we determined AGE levels in macroscopically normal and healthy cartilage collagen. Levels of AGE cross-links were measured as fluorescence (λ_{ex} = 360 nm and λ_{em} = 460 nm), browning (absorption at 340 nm), and pentosidine content. Consistent with our previous data (7), all measures of AGE cross-linking increased with increasing age of the donor. Because collagen AGE levels are greatly influenced by the rate of collagen turnover and collagen turnover is influenced in OA (5), AGE contents were corrected for the possible difference in collagen turnover by expressing the AGE levels per the percentage of $_D$-Asp. This ratio reflects AGE level corrected for collagen turnover (*i.e.* the rate of AGE formation). The rates of formation of AGE fluorescence and browning were substantially higher in the macroscopically normal cartilage than in control cartilage. Fluorescence in the macroscopically normal cartilage collagen was 23% higher than in control cartilage, and browning was 28% higher ($p < 0.01$ in both cases) (Figure 1). Pentosidine per the percentage of $_D$-Asp was 8% higher in macroscopically normal

cartilage compared with control cartilage, but this difference did not reach statistical significance.

RAGE expression. Since AGE accumulation may affect cell function via specific receptors, the qualitative expression of RAGE on FLS and human articular cartilage was tested by RT-PCR. RAGE mRNA was present on FLS from OA (as well as RA) patients (Figure 2A). In addition, RAGE mRNA was found in human cartilage of OA patients and healthy subjects (Figure 2B). However, the variation between the 2 control samples (Figure 2B) was too large for us to draw a conclusion on whether RAGE expression is increased or decreased in OA cartilage based on these results. However, a study reported by Loeser *et al* (32) showed that RAGE expression is higher in OA cartilage than in healthy cartilage.

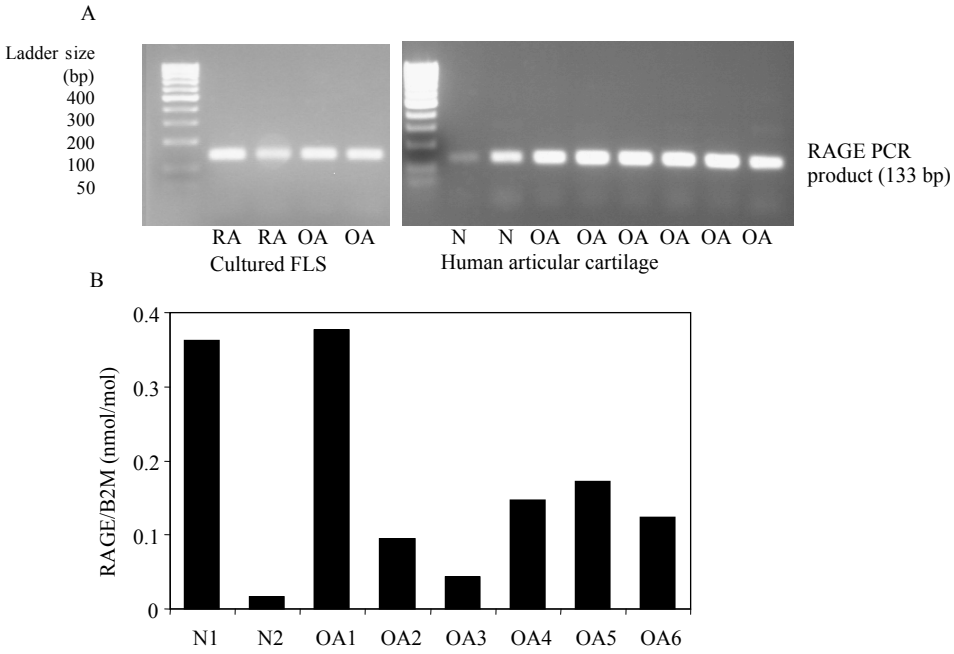

Figure 2. *Presence of receptor for advanced glycation end products (RAGE) in cultured fibroblast-like synoviocytes (FLS).* ***A,*** *RAGE mRNA was present in cultured FLS from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) and in human articular cartilage from OA patients and normal donors (N).* ***B,*** *Amount of RAGE per β2-microglobulin (β2m) mRNA in human articular cartilage from OA patients and normal donors. Values are the mean. PCR = polymerase chain reaction.*

RAGE activation in chondrocytes. Previous studies have found an effect on cell metabolism after glycation of total healthy cartilage explants containing both the chondrocyte and its matrix (10;33;34). From those studies, it is not clear whether these

effects are mediated by changes in matrix stiffness resulting from AGE formation or from direct binding of AGEs to a receptor on the chondrocyte. In order to study the effect of RAGE activation on chondrocytes without confounding factor of matrix glycation, BSA-AGE was added to the culture medium of chondrocytes that were cultured for 3 weeks in alginate beads. Proteoglycan release increased by 13% when chondrocytes were cultured with BSA-AGE compared to chondrocytes cultured without BSA (P<0.001) and by 10% compared to chondrocytes cultured with normal BSA (P=0.004) (Figure 3A). As a result of this increased release, proteoglycan levels in the alginate beads were decreased compared to pre-stimulation levels (Figure 3B). Further support for the catabolic effect of AGEs was provided by the alteration found in the gene expression of MMP-1. MMP-1 per GAPDH mRNA increased significantly compared to control when BSA-AGE was added (385 % P=0.002). A trend in increased MMP-1 per GAPDH levels was found following exposure to nonglycated BSA (170%), although this increase did not reach significance (P=0.09).

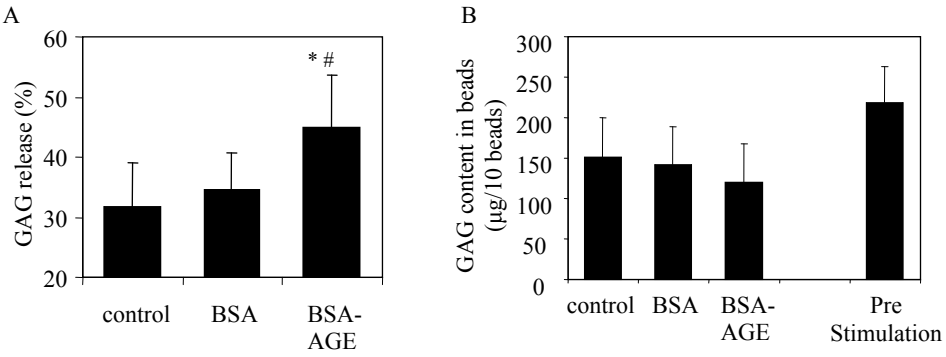

Figure 3. A, *Percentage of glycosaminoglycan (GAG; reflecting the amount of proteoglycan) release by chondrocytes after stimulation with bovine serum albumin (BSA) or glycated BSA (BSA–advanced glycation end product [BSA-AGE]). Upon stimulation with glycated BSA, proteoglycan release was significantly increased compared with control (P < 0.001) or compared with nonglycated BSA–stimulated release (P = 0.004).* ***B,*** *GAG content in beads before and after stimulation. Values are the mean and SD.*

No differences in CII per GAPDH expression was found between the tested conditions. A 300% increase in the ratio MMP-1 per CII mRNA was found upon stimulation with BSA-AGE compared to control cultures (P=0.028) and a 200 % increase in this ratio was found compared with normal albumin (P=0.028) (Figure 4). This ratio was used to obtain a measure for the balance between catabolic and anabolic processes (in other words, the ratio between degradation and synthesis of matrix products).Together, these data indicate that stimulation of chondrocytes by AGEs leads to an increased catabolic processes.

The effect of RAGE activation on FLS invasiveness. Since OA is a disease of not only the cartilage, but of the whole joint, and mild synovial inflammation and hyperplasia are seen in some patients, accompanied with increased MMP production by FLS (20), the effect of AGEs on synovial fibroblast activation was also tested. Invasion experiments were performed to examine RAGE-mediated activation of catabolic processes in FLS (Figure 5). FLS seeded on glycated Matrigel were statistically significantly more invasive (150%; n=6) than cells seeded on control Matrigel (set at 100%; n=6 (P=0.001)). Adding a neutralizing antibody against RAGE statistically significantly reduced invasion back to 115% (n=6) (P=0.012 compared with glycated Matrigel), while it had no effect on basal invasiveness (100%, n=6) (P NS). Isotype control antibodies had no effect on invasiveness either on basal invasiveness or on stimulated invasiveness (both n=4) (P NS).
In order to test whether the AGEs had to be matrix-related, BSA was glycated with 10 mM $_D$-ribose, and added in the upper compartment of the invasion experiment. FLS were significantly more invasive when glycated BSA was added (140%; n=8) (P=0.010) than when control BSA was added. Again, adding a neutralizing antibody against RAGE significantly reduced invasiveness back to baseline (95%; n=8) (P<0.05), and no effect of isotype control was seen (n=4) (P NS for both basal and stimulated invasiveness).

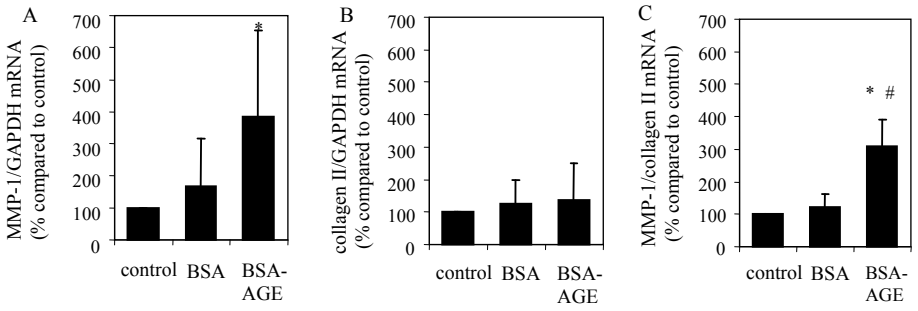

Figure 4. A, *Percentage of matrix metalloproteinase 1 (MMP-1) mRNA production as compared with control and normalized to GAPDH. * = P = 0.002 versus control.* ***B,*** *Percentage of type II collagen mRNA production as compared with control and normalized to GAPDH.* ***C,*** *Percentage of MMP-1 mRNA production as compared with control and normalized to type II collagen. * = P = 0.028 versus control; # = P = 0.028 versus nonglycated BSA–stimulated production. Values are the mean and SD. See Figure 3 for other definitions.*

Activation of RAGE on FLS and protein levels of MMPs. To further study the effects of RAGE activation on FLS, we measured active and pro-MMP-1 levels in the medium of stimulated FLS. FLS were stimulated by normal (non-glycated) BSA or with glycated BSA, and the neutralizing antibody against RAGE was added to see whether the effect was mediated by RAGE (Figure 6). In all donors, pro-MMP-1 was upregulated after stimulation with glycated BSA. FLS stimulated with glycated BSA produced more MMP-

1 than FLS stimulated with normal BSA (240%; n=9) (P<0.001). Addition of a neutralizing antibody against RAGE inhibited this increased MMP-1 production to 145% of that by FLS stimulated with nonglycated BSA (n=9) (P=0.018). Together, these results indicate that RAGE activation on FLS leads to increased catabolic activity.

Discussion

Accumulation of AGEs is a proposed mechanism for the age-related development of OA (4;6;9;11;35). The current study shows that still-healthy cartilage of patients with focal degenerative cartilage lesion elsewhere in the joint has higher AGE levels than healthy cartilage from controls in which there are no signs of OA. The age-related accumulation of AGE crosslinks presents a putative molecular mechanism whereby age contributes to the risk of developing OA. Accumulation of AGEs is however, not only age-related.

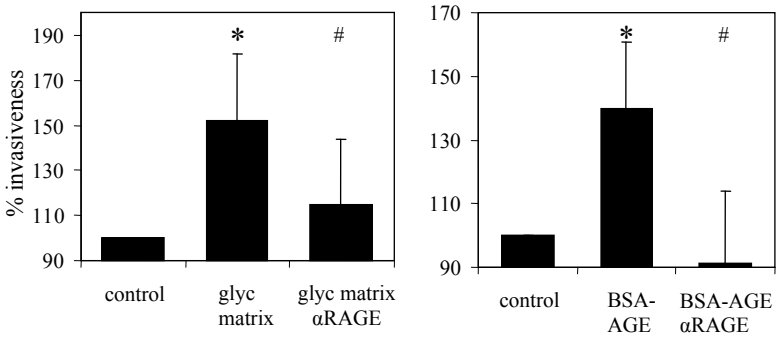

Figure 5. *Activation of osteoarthritis (OA) fibroblast-like synoviocytes (FLS), as determined by their ability to invade a Matrigel matrix.* ***Left,*** *OA FLS were significantly more invasive through glycated Matrigel (* = P = 0.001 versus control). Adding a neutralizing antibody against receptor for advanced glycation end products (αRAGE) significantly reduced invasion (# = P = 0.012 versus glycated Matrigel).* ***Right,*** *AGEs did not have to be matrix related. Addition of BSA-AGE significantly increased invasion through a nonglycated matrix * = P = 0.010 versus control). Increased invasiveness by AGEs was significantly inhibited by an antibody against RAGE (# =P = 0.005 versus BSA-AGE). No effect of nonglycated BSA was seen (data not shown). Values are the mean and SD. See Figure 3 for other definitions.*

In diabetic patients AGE levels tend to be increased, since the hyperglycemia accelerates AGE formation (36). The correlation between diabetes mellitus and OA is supported by some older findings showing that radiographic OA is more common, more severe, and present earlier in patients with diabetes (37-39). In addition, reports from more recent times (in which glaecemic control in patients is much better) still show a trend in the correlation of OA with diabetes (40;41). Thus, OA correlates with both aging and diabetes. In both aging and diabetes, AGE levels are increased. Therefore, the levels of AGEs might predict the susceptibility for OA.

Elevated AGE levels may trigger catabolic pathways through RAGE, as stimulation of chondrocytes (Figures 3 and 4) and synoviocytes (Figures 5 and 6) with AGEs leads to increased production of MMP-1, increased invasiveness, and increased proteoglycan release. Although the production of MMP-1 mRNA and proMMP-1 might only indicate a change in cell metabolism, which does not always lead to actual active MMP-1 and cartilage degradation, functional assays, such as the invasion assay for FLS and the assay for proteoglycan release by chondrocytes, indicate that indeed RAGE activation induces pathways that lead to the degradation of extracellular matrix.

In addition, in a microarray pilot experiment we found an upregulation of mRNA levels of several bone morphogenetic proteins (BMP3, 5, 6, 7 and 8) in OA FLS stimulated with glycated BSA (data not shown). Previous studies have already shown upregulation of BMPs 2 and 6 in FLS by proinflammatory cytokines (42;43). Increased BMP expression might favor osteophyte formation; expression of several BMPs has been found in areas of osteophyte formation (44), and addition of recombinant BMP-2 induces ectopic bone formation in rats (45). BMPs have also been implicated as playing a role in synovial behaviour; synovial thickening in OA can be inhibited by inhibition of endogenous BMPs (46). Together, these findings could indicate that OA-FLS can produce BMPs themselves upon activation by AGEs and thereby induce osteophyte formation and synovial hyperplasia.

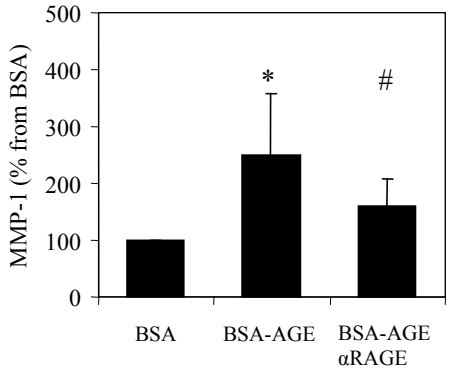

Figure 6. *Pro-matrix metalloproteinase (proMMP-1) production by osteoarthritis fibroblast-like synoviocytes (FLS) after a 48-hour stimulation with glycated BSA (percentage of proMMP-1 productin compared with BSA). Stimulation of FLS with BSA-AGE resulted in significantly increased proMMP-1 production (* = P< 0.001 versus nonglycated BSA). This was inhibited by a neutralizing antibody against receptor for advanced glycation end products (αRAGE) (# = P < 0.05 versus BSA-AGE). Values are mean and SD. See figure 3 for other definitions.*

Activation of catabolic processes after accumulation of RAGE ligands, as we found in chondrocytes and synoviocytes, has been found in several pathologic conditions. An activated catabolic state of cells has been observed in tumors, in which yet another RAGE ligand, S100/calgranulin is able to induce migration and proliferation of the tumor cells (18;47). The major effect of RAGE in tumors seems to be on migration and invasiveness, rather than apoptosis or proliferation, although blockade of RAGE also results in decreased cell proliferation. Increases in levels of MMP-2 and -9 are detected in tumors

with cells bearing wild-type RAGE, but not in those in which RAGE is blocked (48). Furthermore, RAGE triggering can lead to activation of endothelial cells, kidney epithelial cells, and macrophages (14;49;50). The effect of RAGE activation seems to be dependent on cell type and the stage of cell differentiation. In contrast to increased proliferation in tumor cells, AGE stimulation of osteoblasts *in vitro* leads to decreased proliferation and differentiation depending on the differentiation state of the cells (51) and to a decrease in type I collagen production (52). In addition, in osteoporosis, a pathology in which reduced collagen production by osteoblasts plays a role, elevated AGE levels have been found (53). These findings indicate that RAGE activation might in some way play a similar role in bone resorption in osteoporosis and cartilage degeneration in OA.

In conclusion, the activation of RAGE by AGEs leading to cell activation may be one of the molecular mechanisms by which tissue degradation in OA continues. Activation of RAGE by AGEs may lead to an increased MMP production of chondrocytes and synoviocytes, which in turn, induces degradation of cartilage and release of cartilage fragments. Intervention in this vicious circle at the level of both AGE accumulation and RAGE activation may provide good therapeutic targets in the prevention of the ongoing cartilage destruction in OA.

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Chapter 4

The RAGE G82S polymorphism is not associated with rheumatoid arthritis independent of HLA-DRB1*0401

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Rheumatology (2006) 45 (4): 488-490

Sir, The receptor for advanced glycation endproducts (RAGE) has been shown to play a role in several pathologies including Rheumatoid Arthritis (RA)(1). RAGE binding of ligands that are upregulated in RA synovial tissue, fluid and serum, can lead to increased cell activation, including migration, hyperplasia and increased cytokine production. Several animal studies have described a possible role for RAGE in the onset and severity of arthritis. In these animal studies, blockade of the receptor showed suppression of arthritis, while administration of RAGE ligands induced arthritis in healthy mice (2, 3). In-line with these findings, increased levels of RAGE ligands have been found in RA patients and correlate with disease severity (4-6).

Previous studies have indicated that a gain of function mutation of RAGE correlates with RA. Linkage of RAGE with the HLA-DRB1-DQ region, a region known to associate with RA susceptibility and severity, could account for this correlation. To dissect the possible confounding effects of the HLA-DRB1 region for the possible association of RAGE with RA, we investigated the correlation of a gain of function mutation in RAGE, to HLA-DRB1 alleles and RA.

Three hundred and seventy-seven consecutive RA patients of the Leiden Early Arthritis Cohort, a inception cohort for patients with recent-onset arthritis (7) (mean ± SD age 48 ± 17 yrs, 55 % female) and 535 non-RA controls of the same cohort (57 ± 16 yrs, 67 % female) were included in the analysis (Table I). All RA patients fulfilled the 1987 criteria of the American College of Rheumatology. The study was approved by the local ethics committee and written informed consent was obtained from all patients and controls according to the Declaration of Helsinki. HLA genotyping was available for all patients and controls. In addition to type the participants for the RAGE G82S polymorphism we used PCR followed by an overnight digestion with *Alu*I. The 82S polymorphism results in the formation of an extra *Alu*I restriction site.

Hoffman *et al* (2) reported that the RAGE 82S polymorphism correlated with susceptibility for RA. However, RAGE is in strong linkage disequilibrium with HLA-DR4 (8), in particular HLA-alleles that encode a common shared epitope within the HLA-DRB1 allele. In the population studied by Hoffman *et al*, a linkage was found with DR1*0401 and after correction for this allele, the correlation between the RAGE 82S polymorphism and susceptibility for RA was lost. These data were not conclusive, since the number of patients and controls positive for the RAGE 82S polymorphism were very low (5 out of 95 and 2 out of 134, respectively). Here, we identified 46 out of 377 RA patients and 36 out of 535 controls harbouring the RAGE 82S polymorphism. We found an association between the RAGE 82S polymorphism and RA without correlation for HLA alleles. However, in patients, RAGE 82S was in linkage (with a $p<0.01$) with DRB1*0401 [odds ratio (OR) 6.5, $P<0.0001$]. In controls RAGE 82S was in linkage with

DRB1*0401 [OR 4.43, P<0.00001] and 0901 [OR 5.05, P=0.002]. Correction for presence or absence of these HLA alleles was done by the Svejgaard method (9). When the association of RAGE 82S with RA was corrected for the absence or presence of DRB1*0901, the association between RAGE and RA remained present. Coversely, after correction for the presence/absence of HLA DRB1*0401 the association between RAGE 82S and RA was lost (table 2), indicating that RAGE is not associated with RA independently of HLA DRB1*0401 in this cohort. Also in logistic regression analysis with DRB1*0401 and RAGE as possible explanatory variables and the presence of RA as dependent variable, only DRB1*0401 was independently associated with RA (OR 2.5, P<0.001).

In conclusion, considering the size of our study, it is unlikely that the RAGE 82S polymorphism is associated with RA independently of HLA DRB1*0401. However, although this alternation in the receptor itself does not seem to play an important role in RA pathology, the correlation between RA severity and levels of RAGE ligands could still indicate that RAGE ligands do play an important role in RA pathology.

Table 1. *Frequencies of patients and controls positive or negative for RAGE 82S or HLA-DRB1*0401.*

RAGE 82S	DRB1*0401	Patients	Controls
+	+	36	20
+	-	10	16
-	+	78	60
-	-	253	439

Table 2. *Statistical caltulation by the method described by Svejgaard et al. (1)*

comparison	patients		entries of 2x2 table	controls		OR (CI.)	p	Test
RAGE 82S$^+$ vs. RAGE 82S$^-$	*82S+*	*82S-*		*82S+*	*82S-*			
	46	331		36	499	1,99 (1,19-3,12)	0,0045	1) RAGE associated?
DRB1*0401$^+$ vs DRB1*0401$^-$	*DRB1*0401+*	*DRB1*0401-*		*DRB1*0401+*	*DRB1*0401-*			
	11	263		80	455	2,47 (1,76-3,45)	<0,001	2) DR0401 associated?
82S$^+$/0401$^+$ vs 82S$^-$/0401$^+$	*82+/0401+*	*82-/0401+*		*82+/0401+*	*82-/0401+*			
	36	78		20	60	1,38 (0,70-2,77)	0,32	3) RAGE associated in DR040-positives?
82S$^+$/0401$^-$ vs 82S$^-$/0401$^-$	*82+/0401-*	*82-/0401-*		*82+/0401-*	*82-/0401-*			
	10	253		16	439	1,08 (0,45-2,57)	0,84	4) RAGE associated in DR040-negatives?
82S$^+$/0401$^+$ vs. 82S$^+$/0401$^-$	*82-/0401+*	*82-/0401-*		*82-/0401+*	*82-/0401-*			
	36	10		20	16	2,88 (1,00-8,45)	0,029	5) DR0401 associated in RAGE-positives?
82S$^-$/0401$^+$ vs. 82S$^-$/0401$^-$	*82+/0401+*	*82+/0401-*		*82+/0401+*	*82+/0401-*			
	78	253		60	439	2,26 (1,53-3,32)	<0,001	6) DR0401 associated in RAGE-negatives?
Association between RAGE and DR0401 in patients	*patients 82S+*	*patients 82S-*		*patients 0401+*	*patients 0401-*			
	36	10		78	253	11,68 (5,28-26,4)	<0,001	7) Linkage disequilibrium in patients?
Association between RAGE and DR0401 in controls	controls 82S+	controls 82S-		controls 0401+	controls 0401-			
	20	16		60	439	9,15 (4,26-19,74)	<0,001	8) Linkage disequilibrium in controls?

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Chapter 5

RAGE activation induces invasiveness of RA-FLS

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submitted for publication

Abstract:

Objective*: Ligands for the receptor for advanced glycation endproducts (RAGE) are increased in RA synovial fluid (SF), serum, and synovium. Since RAGE is produced by fibroblast-like synoviocytes (FLS), the present study investigates whether the RAGE ligands HMGB-1 and AGEs are able to stimulate the characteristic, pathological invasive behaviour of these cells.*

Methods*: FLS were obtained at joint replacement surgery. FLS were seeded in serum free medium with HMGB-1 or glycated albumin (BSA-AGE) on transwell filters coated with Matrigel. The lower compartment contained medium with serum as a chemoattractant. After three days the percentage of invaded cells was determined and compared to control invasion.*

Results*: Stimulation with HMGB-1 increased invasiveness to 125% compared to control (P=0.001). Addition of anti-RAGE antibody reduced this back to baseline (98%, P=0.002). Stimulation with BSA-AGE, another RAGE ligand, increased invasiveness to 150 % compared to control (P=0.003). Addition of anti RAGE was again able to reduce the increased invasiveness back to baseline (95 %, P=0.008).*

Conclusion*: HMGB-1 and BSA-AGE stimulated the invasiveness of RA-FLS by activation of RAGE. As such, RAGE may be an interesting target for therapy directed at the inhibition of synoviocyte activation.*

Introduction:

In rheumatoid arthritis (RA) the levels of several ligands of the receptor for advanced glycation end products (RAGE) are increased in synovial tissue, synovial fluid (SF), and serum. The first identified group of RAGE ligands, advanced glycation end products (AGEs), are elevated in synovial tissue of RA patients, and also in serum and synovial fluid of RA patients compared to patients with osteoarthritis (OA) and controls (1;2). AGEs are formed by non-enzymatic glycation of exposed amino groups such as on lysine of arginine residues on proteins. AGE formation is accelerated in conditions of hyperglycaemia and/or oxidative stress (3). Levels of a second RAGE ligand, high mobility group box 1(HMGB-1) are increased in synovial fluid of RA patients compared to patients with OA (4;5). *In vivo*, intra-articular injection of HMGB-1 results in an overall frequency of arthritis in 80 % of the animals of different mouse strains (6). Levels of S100A12, a third RAGE ligand, are increased in synovial tissue and serum of RA patients compared to control. S100A12 is found in higher amounts in patients with active arthritis than in patients in clinical remission (7).

In the rheumatoid joint, RAGE is present on a variety of cell types, such as fibroblast-like synoviocytes (FLS), macrophages, T-cells, and certain B-cells (1;8). One of the RAGE ligands, HMGB-1, induces cytokine production by macrophages and can itself be released by macrophages after stimulation with cytokines such as TNF-α (5). However, the FLS in

the inflamed arthritic synovium (pannus tissue) show characteristics similar to cells in pathologic conditions such as tumor growth and diabetes, in which RAGE triggering leads to cell activation, proliferation, and migration. Therefore, our current study explores if RAGE triggering by HMGB-1 or AGEs can lead to enhanced invasion of RAFLS directly.

Material and methods

Synovial fibroblasts

Synovial fibroblasts were obtained at joint replacement surgery or synovectomy after subjects had given informed consent. Tissue was harvested by an orthopedic surgeon and collected in sterile phosphate buffered saline (PBS). Fat and connective tissue were removed and tissue was digested with collagenase (CLS2, Worthington Biochemical Corporation) for at least two hours at 37 ºC. Cells were then separated from the undigested tissue using a 200µm filter (B-Braun Medical, Oss, the Netherlands) and cultured in 162 cm^2 culture flasks (Costar, Cambridge, NY, USA) with Iscove's Modified Dulbecco's medium (IMDM; Biowitthaker) supplemented with glutamax (Biowhittaker, Versiers, Belgium) and 100U/ml penicillin and streptomycin (Biowitthaker, Versiers, Belgium) and 10% foetal calf serum (FCS, GibcoBRL) at 37ºC in a humidified atmosphere of 5% CO_2 in air. At reaching confluence, cells were detached with 0.25% trypsin and split in a 1:3 ratio. For all experiments third passage synovial fibroblasts were used.

Invasion experiments

Invasion assays with FLS were performed using transwells (with 6.5 mm diameter polycarbonate filters with 8.0 µm pore size) coated with 37.5 µg/well Matrigel (Becton Dickinson, USA) to achieve a barrier for the cells to invade through. 4 mg/ml bovine serum albumin (BSA, Sigma-Aldrich) was incubated in IMDM with 10 mM $_D$-ribose (Sigma-Aldrich) at 37 ºC for 3 days to induce glycation (resulting in BSA-AGE) and subsequently dialysed to remove excess ribose. Cells were seeded in the inner compartment of the transwells in serum-free medium with or without HMGB-1 500 ng/ml) or BSA-AGE and in the presence or absence of 2.5 ng anti-RAGE antibody (Santa Cruz, CA, USA). The outer compartment was filled with medium containing 10 % FCS and 10 % human serum to provide a chemoattractant. After three days, the cells were fixed with 4% formaldehyde and stained with a crystal violet solution (1% in PBS). Non-invaded cells were removed by cleaning the inner compartment with a cotton bud and the number of cells that had invaded the matrigel and grown through the transwell membrane was counted using a light microscope. Experiments were all performed in triplicate.

Statistical analysis

Wilcoxon tests were performed in order to test differences between conditions. *P* values < 0.05 were considered to indicate statistically significant differences.

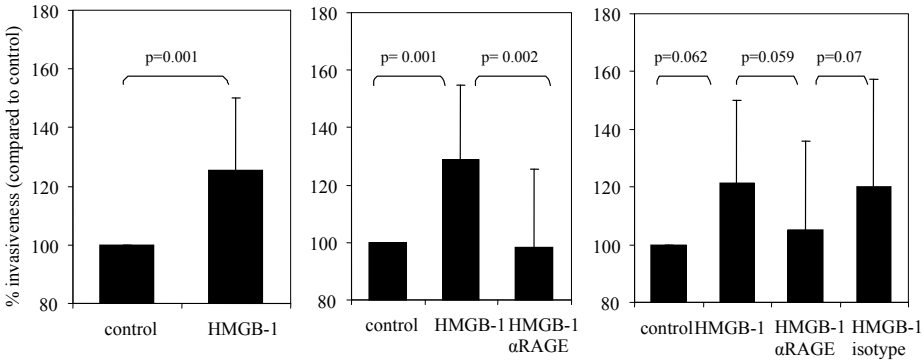

***Figure 1**: Invasiveness of fibroblast-like synoviocytes (FLS) from rheumatoid arthritis (RA) patients, as determined by their ability to invade a Matrigel matrix. FLS were significantly more invasive when HMGB-1 was added (p=0.001) (**A**). When anti RAGE antibody was added, the stimulating effect of HMGB-1 was significantly inhibited (p=0.002 vs HMGB-1) (**B**). An isotype control antibody had no effect on HMGB-1 stimulation (p=0.258, compared to HMGB-1) (**C**).*

Results

To investigate whether HMGB-1 had an effect on the invasive behaviour of RA-FLS, 500 ng/ml HMGB-1 was added in the inner compartment of the transwells. Stimulation with HMGB-1 increased invasiveness to 125% as compared with control (n=18, p=0.001) (Figure 1). This was reduced back to baseline when a neutralizing anti-RAGE antibody was added (98%, n=14) (*P*=0.002).

A similar effect was seen after stimulation with glycated albumin (BSA-AGE). Stimulation of FLS with BSA-AGE increased invasiveness to 150 % (n= 11) (*P*=0.003). This increased invasiveness could again be inhibited by an antibody against RAGE (n=9) (*P*=0.008). Addition of an isotype control had no effect on BSA-AGE induced invasiveness (140 %; n=4) (*P* NS).

Together these data indicate that FLS in RA can be triggered by activation of RAGE by HMGB-1 and AGEs. This points to a broader mode of action of HMGB-1 in the pathogenesis of RA as the previously recognized proinflammatory effects on inflammatory cells.

Discussion

In this study we show that RAGE triggering on RA FLS stimulates invasiveness of these FLS. A role for RAGE in RA pathology is suggested by the increased levels of its ligands in RA patients. HMGB-1, S100/calgranulins, and AGEs all accumulate in RA synovial tissue, fluid, or in serum. Levels of these RAGE ligands correlate with disease incidence and severity (2;4;6;9;10). Previously, RAGE has primarily been implicated in the inflammatory disease processes due to its activation of macrophages and phagocytes

(5;6;11;12). Vice versa, inflammatory processes and cells of the immune system potentially play a stimulating role in the formation of RAGE ligands. S100A12, a member of the S100 family, has increased levels in serum and synovial fluid of patients with active RA, and is expressed in the sub-lining layer of the synovium, associated with granulocytes. Two other S100 family members, S100A8 (MRP8) and S100A9 (MRP14) are overexpressed in the lining layer of inflamed synovial tissue in RA (13). A second RAGE ligand, HMGB-1, has increased levels in RA synovial fluid and upon injection can induce arthritis in several mouse strains (4;6). Activation of macrophages can lead to increased release of HMGB-1, which in turn, can activate macrophages itself (5). Finally, production of a third group of RAGE ligands, the AGEs, is accelerated by oxidative stress, a process occurring during inflammation (3). Pentosidine, one of the AGEs, has elevated levels in articular cartilage, serum, synovial fluid and urine of RA patients which associate with disease severity (9;14).

Besides our study, other studies have shown that RAGE can have effects other than the effects on the inflammatory processes of RA. Studies by Hou *et al* and Owen *et al* (8;15) show that β2-microglobulin modified by AGEs induced the release of monocytes chemoattractant protein (MCP-1) by FLS and production of collagen type I by skin fibroblasts respectively, which, similar to our data, indicate a stimulatory effect of RAGE activation on FLS. Together with our data, this suggests that RAGE not only affects inflammation in the arthritic joint, but might influence the altered, aggressive behaviour of FLS.

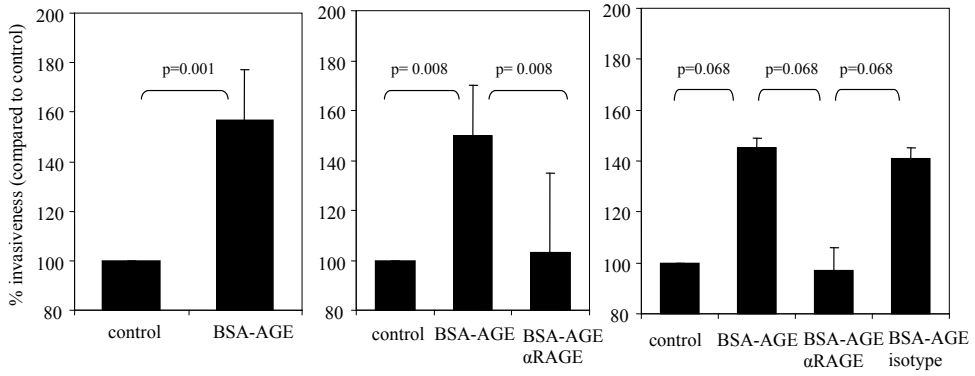

***Figure 2**: Invasiveness of fibroblast-like synoviocytes (FLS) from RA patients, as determined by their ability to invade a Matrigel matrix. Glycated bovine serum albumin (BSA-AGE) significantly increased the invasiveness of the FLS (P=0.003) (**A**). The stimulating effect on invasiveness of BSA-AGE was inhibited by anti RAGE antibody (P=0.008) (**B**). An isotype control antibody had no effect on BSA-AGE stimulation (P=0.273, compared to BSA-AGE) (**C**).*

In conclusion: HMGB-1 and BSA-AGE stimulated the invasiveness of RA-FLS. HMGB-1 and BSA-AGE activated invasiveness could be inhibited completely by an antibody against RAGE, indicating that the HMGB-1 and BSA-AGE effect was RAGE mediated. In combination with the increased HMGB-1 and AGE levels in the SF of RA patients, this suggests that RAGE might be an interesting target for therapy directed at the inhibition of cartilage and bone invasion by pannus tissue.

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Chapter 6

Transition of healthy to diseased synovial tissue in RA is associated with gain of mesenchymal/fibrotic characteristics

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Abstract

The healthy synovial lining layer consists of a single cell layer that regulates the transport between the joint cavity and the surrounding tissue. It has been suggested that abnormalities such as somatic mutations in the p53 tumor-suppressor gene contribute to synovial hyperplasia and invasion in rheumatoid arthritis (RA). In this study, expression of epithelial markers on healthy and diseased synovial lining tissue was examined. In addition, it was investigated whether a regulated process, resembling epithelial to mesenchymal transition (EMT)/fibrosis, could be responsible for the altered phenotype of the synovial lining layer in RA.

Synovial tissue from healthy subjects and RA patients was obtained during arthroscopy. To detect signs of EMT, expression of E-cadherin (epithelial marker), type IV collagen (indicator of the presence of a basement membrane) and α-smooth muscle actin (α-sma; a myofibroblast marker) was investigated on frozen tissue sections using immunohistochemistry. Fibroblast-like synoviocytes (FLS) from healthy subjects were isolated and subjected to stimulation with synovial fluid (SF) from two RA patients and to TGF-β. To detect whether EMT/fibrotic markers were increased, expression of collagen type I, α-sma, and telopeptide lysylhydroxylase (TLH) was measured by real time PCR.

Expression of E-cadherin and type IV collagen was found in healthy and arthritic synovial tissue. Expression of α-sma was only found in the synovial lining layer of RA patients. Stimulation of healthy FLS with SF resulted in an upregulation of α-sma and TLH mRNA. Collagen type I and TLH mRNA were upregulated after stimulation with TGF-β. Addition of BMP-7 to healthy FLS stimulated with SF inhibited the expression of α-sma mRNA.

The finding of E-cadherin and type IV collagen expression in the lining layer of healthy and arthritic synovium indicates that these lining cells display an epithelial-like phenotype. In addition, the presence of α-sma in the synovial lining layer of RA patients and induction of fibrotic markers in healthy FLS by SF from RA patients indicate that a regulated process comparable to EMT might cause the alteration in phenotype of RA-FLS. Therefore, BMP-7 may represent a promising agent to counteract the transition imposed on synoviocytes in the RA joint.

Introduction

Although it has been described that the synovial lining is a mesenchymal tissue because it lacks several epithelial properties, such as tight junctions and desmosomes (1), its function and morphology resemble that of epithelial tissues. Epithelial tissues cover or line body surfaces, forming the surface of the skin, the epidermis, the linings of body cavities (mesothelium), and internal lining of the digestive system and glands. The function of epithelium is to form a barrier between the external and internal environment, and to regulate transport between the cavity it encloses and the adjacent tissue, by facilitating

transport and secretion (2). In normal state, the function of the synovial tissue is to facilitate skeletal movement by the maintenance of a fluid-filled space around cartilage or tendon surfaces. The fibroblast-like synoviocytes (FLS or synoviocytes) are responsible for the excretion of factors such as hyaluronan into the synovial fluid, for clearance of intra-articular debris and regulation of immunological events (1;3).

In rheumatoid arthritis (RA) synovial hyperplasia and inflammation play a prominent role. While influx of inflammatory cells such as macrophages are important in the inflammation of the tissue (4), proliferation of fibroblast-like synoviocytes seems to be a major cause for the hyperplasia of the synovial tissue (5;6). In addition, the data indicate that FLS could play a role in cartilage degradation, as they are found at sites of cartilage degradation in RA and are able to degrade and invade cartilage when co-implanted into a SCID mouse (7).

It has been proposed that the changes in the RA synovium are a random process, caused by e.g. altered expression of p53 (8;9). In RA, overexpression of p53 has been found, often together with somatic mutations in the p53 gene, some of which result in an inactive p53 protein (10-12). For these reasons, it has been suggested that mutations in genes involved in the control of cell-cycle and survival, like p53, are involved in the deranged behaviour of the FLS in RA synovium.

The changes that occur in the synovial lining during development of RA however, resemble the changes of the peritoneal lining during chronic ambulatory dialysis. During chronic ambulatory dialysis, the epithelial cells which form the peritoneal lining become hyperplastic and show a transformed mesenchymal phenotype (myofibroblast phenotype). These changes are induced in a process called epithelial-to-mesenchymal transition (EMT) (13).

Fibrosis, abnormal wound healing, is another process in which EMT can play a role. Myofibroblasts, expressing α-smooth muscle actin (α-sma), are formed from fibroblasts or from epithelial cells by EMT. The myofibroblasts are responsible for matrix deposition and wound contraction and after normal wound healing will die by apoptosis or transform into quiescent cells (14;15). During fibrosis however, the presence of myofibroblasts persists, leading to overproduction of extracellular matrix, and eventually to loss of function of the organ (16). This accumulation of extracellular matrix is also found in RA, indicating that both the gain of invasiveness of RA synoviocytes, as well as the increased matrix production seen in fibrosis represents important pathophysiological events mediated by synoviocytes.

During EMT, the intercellular adhesion molecule E-cadherin appears to play a central role. Transfection of a mesenchymal cell line with E-cadherin resulted in the transdifferentiation into a epithelial cell, while inhibition of E-cadherin expression leads to transdifferentiation from epithelial cells to mesenchymal cells (17). Together with

alteration of cell morphology, other mesenchymal characteristics, such as an invasive motility and the expression of α-smooth muscle actin (α-sma) and vimentin, appear (18). The invasive motility plays an important role in metastasis of tumors into surrounding tissues, a process resembling invasion of FLS into cartilage.
Plasticity resulting from cells shifting between mesenchymal phenotypes is discernible either by EMT only or by the reverse process called mesenchyme-to-epithelium transition (MET). Both processes have emerged as a fundamental principle for reprogramming of gene transcription and as a major determinant of stem cell fate in development and in tissue homeostasis. While TGF-β has been identified as one of the main inducers of EMT during development and fibrotic disorders, another member of the TGF-β super family, bone morphogenetic protein 7 (BMP-7) or osteogenic protein 1 (OP-1), is involved in the maintenance of the epithelial phenotype by induction of MET (19).
Although transforming growth factor β (TGF-β) is the most well known inducer of EMT, EMT can also be induced by S100 calgranulins and advanced glycation endproducts (AGEs) or by the proteolytic digestion of the basement membrane (20-24). Levels of both TGF-β, AGEs and S100 calgranulins are increased in the synovial fluid of RA patients, and therefore the synovial lining is exposed to inducers of EMT (20;25).
The aim of this study was to investigate whether the synoviocytes from RA patients may have undergone a regulated process resembling EMT/fibrosis (as was previously suggested by Zvaifler (26)). This is also of relevance as recent *in vivo* and *in vitro* studies have shown that renal fibrosis can be reversed by administration of bone morphogenetic protein 7 (BMP-7, also called OP-1) (24), indicating that in case EMT plays a role in RA, BMP-7 might be an interesting therapeutic drug.

Material and methods

Patients, human tissue samples and synovial fluid. Synovial tissue was obtained from healthy subjects and RA patients during arthroscopy of the knee. All RA patients met the 1987 criteria of the American College of Rheumatology. Healthy subjects were people that underwent arthroscopy because of ruptured menisci or ligaments. None of these healthy subjects had a history of inflammatory joint disease. Before healthy synovial tissue was collected, permission according to the international conference on harmonisation in Helsinki was obtained. Synovial tissue was obtained at two different hospitals; at both locations permission of authorized ethical commissions was obtained. All patients and healthy subjects gave informed consent. Synovial tissues were collected during the arthroscopy, frozen in Tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, the Netherlands) and cut into 5 µm slices using a cryotome (Leica CM 1900).

In addition, some of the healthy synovial tissue was digested with collagenase IA (1mg/ml Sigma, St. Louis, MO, USA) directly after arthroscopy. Cells were separated from tissue debris by filtration through a 200 µm filter (NPBI, Emmer-Compascuum, the Netherlands) and cultured in 25 cm^2 flasks (Cellstar, Greiner, Alphen aan de Rijn, the Netherlands) with Iscove's Modified Dulbecco's medium (IMDM; BioWhittaker, Versiers, Belgium) supplemented with glutamax (GibroBRL, Paisley, UK) and penicillin and streptomycin (Boehringer, Mannheim, Germany) with 10% foetal calf serum (FCS; GibcoBRL) at 37 ºC in the presence of 5 % CO_2. When cells had reached confluence, they were detached with 0.025 % trypsin and split in a 1:3 ratio. Fourth passage cells were used for stimulation experiments and consisted of more than 95 % FLS as evaluated with light microscopy.
Synovial fluid (SF) was obtained from swollen joints from RA patients. SF was collected in a 50 ml tube, cells were removed and aliquots of SF were kept at -80 ºC. For experiments, SF was diluted with medium.

Haematoxylin eosin staining. Sections from all tissues were first stained with haematoxylin and eosin to evaluate the morphology of the synovial tissue.

Immunohistochemistry of human cryo-sections. Immunohistochemistry was performed following standard protocols. In short: samples were thawed for 30 minutes at room temperatures and then fixed with acetone for 10 minutes. During acetone dehydration, endogenous peroxidase was blocked with 0.3 % H_2O_2. Before applying the first antibody, samples were blocked with 10 % normal human serum in PBS containing 1% bovine serum albumin (BSA) for one hour. After this blocking step, anti E-cadherin (clone 4A2C7, Zymed, San Francisco, USA) was applied diluted 100x in PBS/1 % BSA and incubated overnight at room temperature. The next day sections were thoroughly washed with PBS. For E-cadherin staining, sections were incubated with a second antibody: rabbit anti mouse (Dako, Heverlee, Belgium) for 30 minutes at room temperature followed by 30 minutes incubation with Alkaline Phosphatase-anti Alkaline Phosphatase (APAAP; Dako). Staining was visualized using a 4 Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphatase (NBT-BCIP) reaction, containing 0.33 mg/ml NBT (Stock: 50 mg/ml in 70 % dimethylformamide; Roche, Mannheim, Germany), 0.17 mg/ml BCIP (Stock: 50 mg/ml in 100 % dimethylformamide, Roche), 3.33 mM Levamisole (Sigma), 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2.6H_2O$ pH 9.5. These sections were incubated for 10 minutes at room temperature and the reaction was stopped in H_2O. Sections were counterstained with 1% light green solution (Sigma). Samples were mounted with glycerol glycine and covered with a coverslip.

For α-sma staining, sections were prepared as for E-cadherin. Alpha-sma antibody (clone 1A4, DAKO Cytomation, Heverlee, Belgium) was biotinylated using the DAKO animal research kit (DAKO ARK, Dako) before application and incubated overnight at 4 ºC. The next day sections were thoroughly washed with PBS and incubated with streptavidin-HRP for 15 minutes at room temperature. Nova Red (Vector Laboratories, Burlingame, CA) was used for HRP detection. Sections were counterstained with Mayers haematoxylin, and mounted and covered as described above.

For collagen IV staining, sections were again prepared as for E-cadherin and α-sma staining, and then incubated overnight with a rabbit anti human antibody against type IV collagen (200µg/ml, Santa Cruz, CA, USA). Next, a biotinylated secondary antibody; goat anti rabbit (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used and subsequently conjugated with streptavidin-HRP and detected using NovaRed.

Double stainings for type IV collagen and FLS marker CD55 were performed after preparation of the sections as described above. Sections were incubated overnight with a rabbit anti human antibody against type IV collagen (200µg/ml, Santa Cruz) and subsequently 2 hours with anti CD55 mouse anti human antibody (clone BRIC 110, CLB, Amsterdam, the Netherlands). Next both a biotinylated goat anti rabbit and an AP labelled goat anti mouse (DAKO) secondary antibody were used. The biotinylated antibody was subsequently conjugated with streptavidin-HRP and detected using NovaRed. In addition, the CD55 staining was detected by NBT/BCIP.

Table I. *Primers for real-time PCR.*

Name	Forward primer	Reverse primer
CollIA2	5'CAAGGACAAGAAACACGTCTGGCTAGGAGAAA 3'	5'CAGGCGCATGAAGGCAAGTTGGGTAG 3'
α-SMA	5'-CGTGTTGCCCCTGAAGAGCAT-3'	5'- ACCGCCTGGATAGCCACATACA -3'
TLH	5'-TTAAAGGAAAGACACTCCGATCAGAGATGA-3'	5'-AATGTTTCCGGAGTAGGGGAGTCTTTTT-3'
β_2M	5'TCTTGTACTACACTGAATTCACCCCCACTGA 3'	5'ATCCAAATGCGGCATCTTCAAACCTC3'
Col IV	5'-GCTCACCAGACCAGTGGGT-3'	5'-TCACCTTTAGGTGCTG-3'

Reverse transcriptase polymerase chain reaction (RT-PCR) on cultured fibroblast-like synoviocytes. FLS from RA patients and healthy controls were plated in a 24-well flat bottom plate at a density of 100,000 cells per well. After 72 hours, cells were lysed for RNA isolation. RNA was isolated using the Qiagen RNeasy kit and subsequently converted to cDNA (AMV reverse transcriptase, Roche, Penzberg, Germany) and subjected to RT-PCR amplification. cDNA was amplified using specific primers for type

IV collagen and the housekeeping gene β2-microglobulin (β_2M) (table I). The total reaction volume of 25 µl contained 1x PCR buffer (Applied Biosystems), 0.4 mM of each dNTP, 2.5 mM $MgCl^{2+}$, 500 nM of each primer and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems) together with 10 µl 5x diluted cDNA. PCR was performed using a iCycler (Bio-Rad laboratories Inc, Hercules, CA, USA) and consisted of a 4-minute interval at 94 ºC followed by 30 cycles of 95 ºC for 30s, 56 ºC or 60 ºC for 45s, and 72 ºC for 30s, for β_2M and type IV collagen respectively. An aliquot of 15 µl of each sample was subjected to electrophoresis in a 2 % agarose gel containing ethidium bromide.

Stimulation of healthy FLS with TGF-β and SF. One day prior to stimulation, healthy FLS were plated in a 24-well flat bottom plate at a density of 100,000 cells per well. One hour before stimulation, cells were washed with PBS and incubated in serum free medium. Cells were stimulated in medium with 0.1 % lactalbumin hydrolysate (as serum replacement) alone (as a control) or in medium with 0.1 % lactalbumin hydrolysate containing either 1 ng/ml TGF-β or 10x diluted synovial fluid for an additional 48 hours. At this point, cells were lysed for RNA isolation. RNA was isolated using the Qiagen RNeasy kit and subsequently converted to cDNA (AMV reverse transcriptase, Roche, Penzberg, Germany), and subjected to real-time PCR amplification. cDNA was amplified using specific primers and specific beacons for collagen type I α2 (CollIA2), α-smooth muscle actin (α-sma), telopeptide lysyl hydroxylase (TLH), and the housekeeping gene β_2M (table I and II respectively). The total reaction volume of 25 µl contained 1x PCR buffer (Applied Biosystems), 0.4 mM of each dNTP, 2.5 mM $MgCl^{2+}$, 500 nM of each primer and beacon and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems) together with 10 µl 5x diluted cDNA. Real-time PCR was performed in an ABI PRISM® 7700 sequence detection system and consisted of a 5-minute interval at 95 ºC followed by 40 cycles of 95 ºC for 30s, 56 ºC for 40s, and 72 ºC for 30s. Data were analysed using Sequence Detector version 1.7 software.

***Table II**. Molecular beacons for real-time PCR. Abbreviations used in this table are: DABCYL,-(4-dimethylaminophenyl) benzoic acid; FAM, 6-carboxyfluorescein.*

Name	Sequence
CollIA2	5' FAM-cgtgccGGCAGCCAGTTTGAATATAATGTTGAAGGAggcacg-DABCYL 3'
α-SMA	5' FAM-cgtcgCCAAGGCCAACCGGGAgAAAATGACgcgacg- DABCYL 3'
TLH	5'-cgtgcgCGTGATAAACTGGATCCTGATATGGCTCTTcgcacg-DABCYL-3'
β_2M	5'FAM- cgtgcCCTGCCGTGTGAACCATGTGACTTTGgcacg – DABCYL 3'

Statistical analysis. For statistical analysis, means and standard deviations were calculated. Differences between conditions were tested for statistical significance using the Kruskall-Wallis test with a *post hoc* Mann-Whitney U-test. Differences were considered statistically significant at $P<0.05$. All statistical analyses were performed using SPSS 11.5 Software (SPSS, Chicago, IL).

Results

Expression of epithelial and mesenchymal markers on synovial tissue. As previously described, staining of human synovial tissue with HE showed an increase in the number of cell layers of the synovial lining of RA patients (figure 1). E-cadherin staining was found in the healthy synovium, especially in the lining layer (figure 2). In arthritic synovial tissue E-cadherin expression pattern was more widely spread, possibly due to hyperplasia (found in all patients). The presence of E-cadherin staining indicates that most synoviocytes display epithelial properties.

Another epithelial property is the presence of a basal membrane, consisting of collagen IV and laminin. Collagen IV was present all along the lining layer of both healthy and arthritic synovial tissue, indicating the presence of a (partial) basement membrane (figure 3A). Double staining with FLS marker CD55 showed co localisation of type IV collagen and CD55 expression (figure 3E-F). In addition, expression of type IV collagen mRNA was found in cultured FLS from both RA patients and healthy controls (figure 3I), indicating that FLS are able to produce type IV collagen.

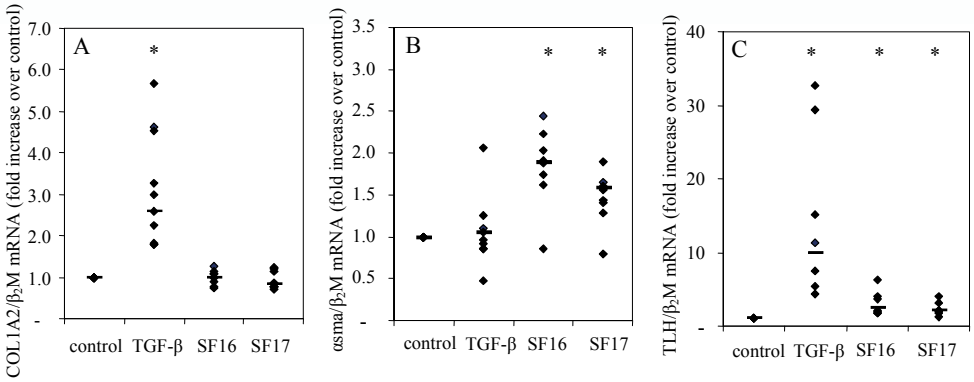
Additional markers were tested to further define the epithelial and mesenchymal properties of the synoviocytes in the lining layer. Alpha-sma is a myofibroblast marker often used to identify myofibroblasts after EMT. In healthy synovium, α-sma expression was only found in blood vessels. In contrast, in synovial tissue from 4 out of 8 patients with RA, α-sma expression was also found in the lining layer, suggesting that the lining of the synovium in RA patient contains myofibroblasts (figure 4). Together these data indicate that several epithelial markers are present in healthy and RA synovial tissue. In addition, the presence of α-sma in human RA synovial tissue indicates that an EMT-like/fibrotic process has occurred.

Figures 1-4 are shown in the appendix (page 145).

Different effects of TGF-β, SF and BMP-7 stimulation on collagen IA2, α-sma, and TLH expression in FLS. To investigate whether synovial fluid is able to induce an alteration in phenotype and expression of healthy FLS, FLS obtained from 10 healthy subjects were stimulated with TGF-β and SF from two RA patients. Expression of three markers often upregulated in fibrotic processes and after EMT, collagen Iα2, α-sma, and

TLH mRNA, was measured and corrected for the expression of β_2M. Upon stimulation with SF from two different RA patients, expression of collagen I remained stable, while expression of α-sma was modestly increased (1.8 ± 0.5 (mean ± SD) fold upon SF16 and 1.4 ± 0.3 fold upon SF17; n=10) (P<0.001 for both SF's) (figure 5). This indicates an alteration from healthy FLS to a phenotype resembling that of differentiated myofibroblasts, a cell type that shows increased proliferation and is capable of migration and invasion. In addition, we found an upregulation in TLH, an enzyme responsible for the increased crosslinking of collagen in fibrotic tissue (27), after stimulation of healthy synoviocytes with SF (3.4 ± 1.7 fold induction upon SF16 and 2.7 ± 1.3 fold upon SF17, n=10) (P<0.001 for both SF's) (figure 5). This indicates that SF might also induce collagen crosslinking in synovial tissue, thereby reducing the susceptibility of collagen for degradation.

Figure 5. *Expression of collagen type I, α-sma and TLH in healthy fibroblast like synoviocytes (FLS) upon stimulation with TGF-β and SF. Expression of collagen type I (COL1A2)/β_2M mRNA (**A**), α-smooth muscle actin (α-sma)/β_2M mRNA (**B**) and telopeptide lysylhydroxylase (TLH)/β_2M mRNA (**C**) were measured after stimulation of healthy FLS with 1 ng/ml TGF-β or 1/10 diluted synovial fluid (SF) from RA patients. TGF-β increased collagen IA2 expression, but had no effect on α-sma expression. Stimulation of healthy FLS with RASF did not lead to an increase in collagen IA2/β_2M but did induce α-sma/β_2M mRNA expression. TLH expression was induced by both TGF-β and SF (–: median, ♦: FLS from individual patients).*

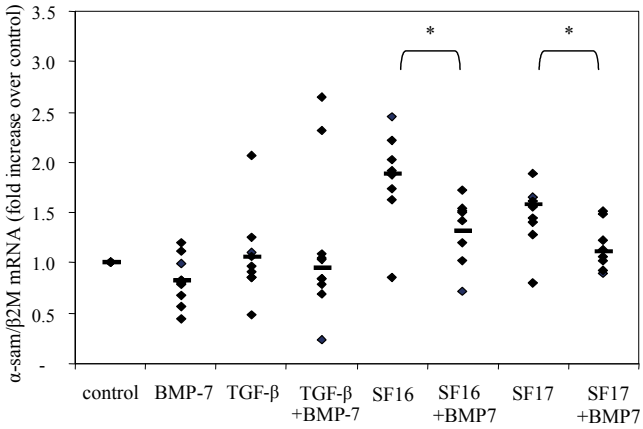


To study whether the effects of SF on α-sma expression could be induced by TGF-β in the SF, healthy FLS were stimulated with 1 ng/ml TGF-β (the level of total TGF-β normally found in RA SF (28)) and expression of collagen I, α-sma and TLH was measured. Interestingly, a discrepancy between stimulation with TGF-β and SF was found. Upon stimulation with TGF-β, an increased expression of collagen I (3 ± 1.4 fold increase; n=10) (P<0.001) and TLH (14.5 ± 10.8 fold increase; n=8) (P<0.001) was found. No effect of TGF-β on α-sma expression was found. This could indicate that although this concentration of TGF-β is able to induce enzymes responsible for modification of

extracellular matrix in healthy FLS, it is unable on its own to induce an alteration towards myofibroblasts after 48 hours.

Using an ELISA for active TGF-β1, we found only very low levels of TGF-β1 in our synovial fluid (data not shown), indicating that indeed the effect of SF on healthy FLS might not be mediated by TGF-β1 in the SF from these two donors. Although the differences found with real time PCR are modest, they are highly reproducible: 10 donors cultured on different days and assayed by real time PCR on different days show similar results when cultured with TGF-β and SF. In addition, culture of cells from 2 patients and subsequent analysis by real time PCR was repeated after a few weeks and again similar results were found. Together these data indicate that even within this relatively short time-frame, SF and TGF-β are able to induce EMT/fibrotic markers in healthy synoviocytes.

Figure 6. *Expression of α-sma upon stimulation with TGF-β or SF in combination with BMP-7 is shown. BMP-7 treatment of healthy FLS inhibited the induction of α-sma mRNA by SF16 and SF17 in FLS from all donors (-: median ♦: FLS from individual subjects).*



BMP-7 has been described as an inhibitor of EMT and fibrosis and can induce expression of epithelial markers. These opposing effects of BMP-7 on TGF-β signalling are of interest as they could inhibit the fibrotic process evoked by SF and/or TGF-β. We aimed to examine whether addition of BMP-7 to synoviocytes obtained from healthy donors would inhibit the expression of the fibrotic marker α-sma (figure 6). Administration of BMP-7 to healthy FLS *in vitro* had no effect on α-sma expression in unstimulated cells (0.8 ± 0.2 fold expression, n=10) (P=0.06) (figure 6). Administration of BMP-7 together with SF inhibited the effects SF, as evidenced by the observation that expression of α-sma was less pronounced (inhibition of 1.8 fold compared to control to 1.3 for SF16 and from 1.4 to 1.2 for SF17; n=10) (P<0.01 for both SFs).

Together, these data indicate that TGF-β and synovial fluid from RA patients are able to induce upregulation of EMT/fibrotic markers on mRNA levels, which can be partially suppressed by BMP-7.

Discussion

This study indicates that the cells of the synovial lining layer not only have same function as epithelium, the formation of a barrier and the regulation of transport between a cavity and the adjacent tissue, but also express E-cadherin, and are located on a (fragmented) basement membrane. Although some studies could not report expression of E-cadherin in synovial tissue (29), our study is in line with a study by Trollmo *et al* (30), describing E-cadherin expression in the lining layer of healthy synovial tissue, indicating the presence of molecules that mediate cell-to-cell adherence. Like in epithelium, the presence of a basement membrane also appears present as type IV collagen, an important constituent of a basement membrane, was readily detectable in the lining layer of healthy synovium and seemingly forming a layer underneath the synovial lining cells (31). Although some mesenchymal markers, such as vimentin and cadherin-11, are also present in the lining and sublining of healthy synovial tissue (29;29;32), expression of α-sma is absent, while expression of the epithelial marker E-cadherin is present. Together with the function; lining of the joint cavity, ultrafiltration of synovial fluid, and excretion of hyaluronan into the synovial fluid, these characteristics indicate that synovial tissue represents an epithelial-like tissue. In synovial tissue from RA patients, E-cadherin staining is in both lining and sublining adjacent to the lining. This same staining pattern is found for type IV collagen. This observation implies that in RA patients, cells from the lining layer start proliferating, but maintain part of an epithelial phenotype when they migrate more into the sublining. Expression of type IV collagen has also been found by others (31;33). Rinaldi *et al* however found a decrease in collagen IV expression, which correlated with the grade of inflammation of the rheumatoid synovia. This decrease in type IV collagen indicates the destruction of a basement membrane, a feature of EMT/fibrosis. In addition they found that type IV collagen was expressed by FLS. However, type IV collagen expression could not be downregulated by TGF-β *in vitro* (33).

The synovial membrane in RA patients shows features of both tumors and fibrotic tissue, as a tumor-like hyperplasia of the synovial lining and a fibrotic deposition of extracellular matrix are present. In diseases such as renal fibrosis and carcinomas, transition of an epithelial phenotype to a mesenchymal phenotype plays an important role in disease pathology, as it alters not only the phenotype, but also the migratory potential of the cells. Although we did not analyse the biological consequences of the alterations induced in FLS after exposure to SF, it has been described that loss of E-cadherin and change in integrin lead to the loss of cell-cell adhesion. In addition, gain of α-sma and the cytoskeletal

rearrangement provide tools for gaining motility. Therefore, we propose that the EMT phase or the number of synovial cells that have undergone EMT is indeed correlated with the infiltration of synovial cells into cartilage. The findings of this study, combined with a previous observation describing that invasiveness of FLS is a patient characteristic, as the difference in invasiveness between patients is greater than the difference between two joints from one patient (34), suggest that a regulated EMT-like process might play a role in development of an arthritic synovium, rather than a random process of tumor like alterations caused by the presence of p53 mutations in RA synovium (8;9;34).

A myofibroblast marker, α-sma, was found in the lining of half of the RA patients, indicating that in these RA patients, synovial cells have a myofibroblastic phenotype, the same cells responsible for collagen accumulation in fibrosis. These data are supported by a finding by Aidinis *et al* who also found stress fibres in RA synovium, indicating that myofibroblasts might be present (35). Our study shows that SF from RA patients is able to induce α-sma expression in cultured healthy FLS. These data indicate that factors present in the synovial fluid from RA patients are able to induce a transformation to myofibroblasts. Although both SF and TGF-β had an effect on the healthy FLS, the mechanism seems to be different. After TGF-β stimulation, primarily type I collagen and TLH expression are upregulated, after SF stimulation, α-sma and TLH expression are upregulated. In the healthy FLS type I collagen expression rather than α-sma expression was induced after stimulation with TGF-β. These findings seem in discordance with a study in which it was shown that cultured FLS from RA patients transformed from α-sma negative cells to α-sma positive cells after stimulation with TGF-β (36). In that study however, only high levels of TGF-β were able to induce α-sma expression. In our study, we used a level of TGF-β previously found present in SF from RA patients (28). Therefore, the use of synoviocytes from healthy individuals combined with lower concentrations of TGF-β to stimulate these synoviocytes might explain the absence of α-sma induction by TGF-β in our synoviocytes. Although in this study TGF-β was not able to induce α-sma mRNA expression, SF was able to induce α-sma expression in the healthy synoviocytes in the relatively short time frame analysed. This indicates that besides TGF-β, other factors can play an additional role in induction of the alteration of phenotype in RA. Although the effect of many cytokines has not been studied yet, it has been described that the formation of myofibroblasts results from a combination of cellular fibronectin extra domain (ED)-A with TGF-β (37). Fibronectin ED-A is an isoform of fibronectin and has elevated levels in RA synovial tissue (38). Therefore, it is possible that TGF-β plays a more important role in patients, because the cellular fibronectin ED-A present in the synovial tissue will enhance TGF-β activity.

In addition, the combination of accumulation of ligands of RAGE in SF from RA patients (20;39-44), and the ability of advanced glycation endproducts (AGEs) to induce EMT in

kidney fibrosis via RAGE triggering independently of TGF-β (22;23), indicates that triggering of the receptor for advanced glycation endproducts (RAGE) could play a role in the induction of EMT/fibrosis in RA patients.
Previously, BMP-7 has been shown to counteract the action of TGF-β in inducing EMT, and to reverse chronic renal injury (24). This study shows that BMP-7 is also able to suppress alteration in FLS induced by SF from RA patients. Therefore, BMP-7 therapy is potentially not only interesting for RA patients for its beneficial effects on cartilage and bone metabolism (45), but also for its role in maintaining a quiescent phenotype of the synovial lining layer.

Conclusions: Like epithelium, the synovial lining layer forms a barrier between a cavity and the adjacent tissue. However, while synovial fibroblasts do express E-cadherin, other features of epithelium, such as the formation of a continuous cell layer, and the presence of tight junctions and desmosomes, are not found. Therefore, the synovial lining can be called epithelial-like rather than a real epithelium. Nevertheless, this does not mean a process resembling EMT/fibrosis cannot occur; EMT has been shown in the endocardium, a tissue also lacking desmosomes (46). In addition, our data shows the presence of E-cadherin and type IV collagen, and supports the finding that synovial tissue can undergo a process resembling EMT/fibrosis. In this process the resting synovial lining layer becomes hyperplastic and acquires an altered phenotype, including an increased invasive mobility, thereby contributing to joint destruction in patients with RA. To inhibit or reverse this process, administration of BMP-7 could be an interesting option for treatment of rheumatoid arthritis.

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Chapter 7

Summary and Discussion

Joint diseases affect a large subset of the world population. The most common joint diseases are osteoarthritis (OA) and rheumatoid arthritis (RA). OA and RA pathology differ in some of the hallmarks. OA is hallmarked by cartilage degradation, with only secondary inflammation of the synovial tissue. It is believed to be a wear and tear disease and is highly associated with aging (over 50% of people over 60 years of age is affected). A prominent age-related change in cartilage is the accumulation of advanced glycation end products (AGEs), that affect the mechanical properties of the cartilage and the turnover by chondrocytes (1-4). RA is hallmarked by an inflamed synovial tissue, cartilage degradation, and bone erosion. RA is an autoimmune disease; in the serum of RA patients several auto-antibodies are found (5). Despite the differences, the net results of the diseases resemble each other; both diseases will eventually lead to loss of function of the joint and disability.

The studies in this thesis are focussed on the behaviour and activation of chondrocytes and the fibroblast-like synoviocytes of OA and RA patients. The first subject in this thesis is the interaction between chondrocytes and synoviocytes. The relative contribution of chondrocytes and synoviocytes was studied in a new in vitro model for cartilage degradation. Due to availability, bovine chondrocytes were cocultured with synoviocytes from RA patients to investigate which cell type is involved in cartilage degradation during RA.

In the second part of this thesis, it is shown that activation of the receptor for advanced glycation end products (RAGE) plays an important role in both pathologies. Activation of chondrocytes was studied as change in expression of type II collagen and MMP-1 and the degradation of cartilage by the chondrocytes after stimulation. Activation of synoviocytes was studied by production of MMP-1 and functionally by an invasion assay. Finally, this thesis describes the synovial tissue of healthy subjects and RA patients. While the synovial lining from healthy subjects show many features of epithelium, the synovial lining from RA patients shares properties of fibrotic/tumorigenic tissue. It is hypothesised that the synovial lining is an epithelial-like tissue and that it can undergo a process resembling epithelial to mesenchymal transition or fibrosis during RA pathology.

Interaction between chondrocytes and synoviocytes in an *in vitro* model for cartilage degradation

During research of RA and OA pathology *in vitro*, usually different mechanisms are studied. In the context of OA pathology, cartilage degradation and production by chondrocytes is mostly studied. In RA pathology, activation of synoviocytes is mostly studied. Increasing evidence however indicates that it is likely that interaction between chondrocytes and synoviocytes is an important factor in both pathologies. In addition, for the study of synoviocytes in RA, often models are used that consist of type IV collagen (like the one used in chapter 3 and 5) or type I collagen. Since not all MMPs are able to

degrade the components of cartilage (such as type II collagen and proteoglycans), it is crucial to include cartilage components in models for cartilage degradation by synoviocytes or chondrocytes. In ***chapter 2*** a model is described which combines bovine chondrocytes with synoviocytes from RA patients. Although human articular chondrocytes would be preferred above bovine chondrocytes, the scarce availability of human articular chondrocytes and their lack of proliferation make the bovine articular chondrocytes more suitable. The bovine chondrocytes have a virtually unlimited availability and are widely accepted and used as a substitute for human articular chondrocytes in *in vitro* studies (6-8). The use of human chondrocytes from the growth plate is rejected because their behaviour differs distinctively from that of articular chondrocytes (9).

Therefore, for this model bovine chondrocytes were isolated and cultured in alginate beads for one week. After this initial week, beads were dissolved and the resulting cell suspension was seeded in transwells. Subsequently, the cells were cultured with ^{35}S containing medium for another week to label the proteoglycans. An additional two weeks of culture followed to allow washing of the excessive ^{35}S and further maturation of the matrix. During these three weeks of culture in transwells, cartilage-like slices were formed, resembling native cartilage biochemically, as was also found by the group of Thonar (7, 8).

After this culture period, the actual experiment was started, in which cartilage-like slices with live or killed chondrocytes were cultured alone, or together with synoviocytes from RA patients. The RA synoviocytes were only able to induce an increase in cartilage degradation in the presence of live chondrocytes. In addition, while supplementation of IL-1β and TNF-α had a stimulatory effect on cartilage degradation of monocultures of live cartilage-like slices, no effect on synoviocytes was seen. These findings indicate that the role for synoviocytes in the rheumatoid joint might depend on their interaction with chondrocytes. Moreover, they show that in studies of invasive behaviour of synoviocytes, the composition of the matrix and the interaction between synoviocytes and other cells within the joint should be taken into consideration.

RAGE activation plays an important role in the behaviour of chondrocytes and synoviocytes during OA and RA.

One of the similarities between OA and RA is the accumulation of ligands for the receptor for advanced glycation end products (RAGE). In OA cartilage, increased levels of advanced glycation end products (AGEs) and increased expression of their receptor RAGE by chondrocytes is found (10-12). AGEs are formed during non-enzymatic glycation of proteins. The AGEs accumulate in long-lived proteins such as cartilage collagen because once they are formed, they cannot be removed unless the whole protein is removed. Both aging and levels of AGEs in cartilage predispose to the development of

OA (13, 14). Since AGE levels correlate with age, it is likely that the age related increase in AGEs might be responsible for the increased susceptibility for OA.
Possible mechanisms by which AGEs increase susceptibility for OA are described in previous studies (1-4). It is shown that accumulation of AGEs in cartilage leads to an alteration in cartilage stiffness, which may contribute to failure to resist damage. In addition, accumulation of AGEs affects the cartilage metabolism, leading to decreased proteoglycan synthesis and collagen turnover (1, 3). These changes in metabolism could be caused by the altered properties of the cartilage matrix, or directly by binding of AGEs to receptors for AGEs, such as AGE-R1, AGE-R2 and RAGE, present on chondrocytes. In ***chapter 3*** a study is described in which the role for AGEs and RAGE in OA pathology is further investigated. To elucidate whether AGE levels might predispose to OA, visually intact cartilage from patients with focal degenerative cartilage elsewhere in the joint (N_{DEG}) and cartilage from age matched individuals without any sign of cartilage degeneration (controls) were analysed for AGE levels and collagen turnover. Collagen turnover was already increased in the N_{DEG} cartilage. Therefore, AGE levels were corrected for the residence time of collagen. N_{DEG} cartilage contained significantly more AGEs than control cartilage. These results indicate that increased AGE levels might precede actual cartilage damage, and could therefore predispose for the development of OA. To investigate the process by which AGEs could influence cells involved in OA pathology, chondrocytes and synoviocytes were stimulated with AGEs. This study reveals that chondrocytes stimulated with AGEs which are not matrix-bound have more catabolic activity, resulting in the degradation of the cartilage matrix. This is in contrast to earlier studies that show a decreased cartilage release after glycation of the matrix. Since AGE formation in cartilage affects the susceptibility for MMP-mediated degradation, this discrepancy might be because in our study the matrix itself remained unchanged, while in the previous studies the chondrocyte matrix itself was glycated.
In addition to alteration of chondrocyte activity, alteration in synoviocyte activity was found. Upon stimulation with AGEs, synoviocytes from OA patients produced more (pro)MMP-1 and showed increased invasiveness through a Matrigel matrix. Increased AGE levels in OA thus can have effects on both chondrocytes and synoviocytes in the joint, leading to increased cartilage degradation by activation of RAGE.

Among the several polymorphisms for RAGE that have been identified, the RAGE 82S polymorphism is most frequently studied because it induces an amino acid change. The RAGE 82S polymorphism is a functional polymorphism in the ligand binding domain. Functional studies have indicated that cells heterozygous for the polymorphism exhibit increased activity upon ligand binding compared to cells without the polymorphism (15). In addition, Hoffman *et al* indicated a possible role for this RAGE 82S polymorphism in the susceptibility for RA. RAGE 82S correlates with RA. However, since RAGE is

located on the same chromosome as the HLA alleles, a correction has to be made for the presence of SE alleles. Due to small patient numbers positive for the polymorphism, the correlation between RAGE and RA was lost after correction for HLA DRB1*0401 (the SE allele with which the RAGE 82S polymorphism was correlated). Our study of RAGE 82S polymorphism in the large Leiden Early Arthritis Clinic is described in ***chapter 4***. Considering the size of the cohort in our study (377 patients and 535 non-RA controls), we concluded that it is very unlikely that the RAGE 82S is associated with RA independently of HLA DRB1*0401.

Although this functional polymorphism might not have an effect on susceptibility for RA, other data indicate that activation of (wild type) RAGE does have an effect. In RA, increased levels of several RAGE ligands have been found. Pentosidine (one of the AGEs), S100/calgranulins, and high mobility group box 1 (HMGB-1) all show increased levels in the synovial fluid, - tissue, and serum of RA patients (16-22). Disease activity correlates with levels of pentosidine and S100A12, indicating an important role for RAGE ligands in RA (16, 20). While a role for HMGB-1 stimulation of macrophages has been described in previous studies, we investigated the effect of HMGB-1 and AGEs on synoviocytes from RA patients. The results in ***chapter 5*** show that stimulation of synoviocytes by HMGB-1 or glycated albumin leads to increased invasiveness through a Matrigel matrix. This effect could be inhibited by a neutralizing antibody against RAGE (α-RAGE), indicating that activation of the synoviocytes by HMGB-1 or AGEs was RAGE-mediated. These data indicate that increased AGE and HMGB-1 levels in RA synovial tissue en -fluid can activate not only the inflammatory process as described previously, but can also activate cartilage degradation by RA synoviocytes.

Synoviocytes from the lining layer of RA synovium undergo a process resembling EMT/fibrosis

The hypothesis studied in ***chapter 6*** is that in healthy subjects the fibroblast-like synoviocytes (FLS) from the synovial lining layer are epithelial-like cells that undergo a process resembling epithelial to mesenchymal transition (EMT)/fibrosis during RA, leading to thickening of the synovial lining and invasive behaviour of the fibroblast-like synoviocytes. The hallmarks of epithelial tissue are the barrier function and the structured morphology with numerous junctions. Epithelium forms the lining of body surfaces and cavities, as well as glands. It functions as a barrier and regulates transport between the environment and the adjacent tissue. The synovial lining has a similar function; it forms a barrier between the joint cavity and the joint capsule, and is responsible for the ultrafiltration of the synovial fluid from blood and the production and release of matrix proteins into the synovial fluid. Although the synovial lining layer lacks some of the junctions present in epithelium, gap junctions (facilitating communications between cells) and desmosomes have been found. In addition, our study shows presence of E-cadherin,

the major component of adherens junctions, in the synovial lining of healthy subjects and RA patients. As described before, another important feature of epithelium, the presence of a (fragmented) basement membrane (consisting of type IV collagen and laminin) is found just under the lining layer. Except for a thickening of the cell layers positive for E-cadherin, no differences were found between E-cadherin and type IV collagen expression between healthy subjects and RA patients. Interestingly, expression of a myofibroblast marker, α-smooth muscle actin (α-sma) was only found in the lining layer of RA patients, indicating an alteration in phenotype. Results from further i*n vitro* studies show that synovial fluid of RA patients was able to induce the expression of two EMT/fibrotic markers; α-sma and TLH, in synoviocytes obtained from healthy subjects. Synovial fluid did not have an effect on the expression of type I collagen (the major collagen produced during fibrosis). TGF-β, a know inducer of EMT/fibrosis induced the expression of type I collagen and TLH, but not of α-sma, indicating that a different mechanism could be involved. BMP-7 has been described to reduce fibrotic processes in an *in vivo* model for renal fibrosis. Addition of BMP-7 to healthy synoviocytes stimulated with synovial fluid resulted in an inhibition of α-sma induction. It is therefore interesting to further study BMP-7 as a therapeutic compound, not only because of it has stimulating effects on cartilage synthesis, but also because it induces a more quiescent phenotype in synoviocytes.

Conclusion and perspective

Although the pathologies of OA and RA differ, the effect of RAGE activation on synoviocytes and chondrocytes might be an interesting similarity for development of new therapeutic treatments.

In the present work evidence for the role of RAGE activation in increased cartilage degradation by chondrocytes and synoviocytes is provided. Several other publications indicate a role for RAGE in the activation of macrophages in RA, additional to the role in cartilage degradation. Therapies for RA usually address the inflammatory pathways, reducing only the inflammation in the joint, while a clear effect on cartilage degradation is not seen. Since the data from this thesis and studies of macrophages show that RAGE can activate a broad range of cells within the joint, it might be more interesting to intervene in the RAGE pathway.

This intervention in the RAGE pathway could be done by reducing the increased levels of RAGE ligands, or by blockade of RAGE. Reducing AGEs in the OA cartilage and levels of AGEs, HMGB-1, and S100/calganulins in synovial tissue and fluid would be more complicated because several different processes can be involved in the production. Therefore, blockade of RAGE, which will block the effect of all RAGE ligands, might be a more logical and practical therapy.

By blocking the RAGE pathway, both inflammation (by reducing macrophage activation) and cartilage degradation (by chondrocytes and synoviocytes) can be addressed. *In vivo* studies in mice with collagen-induced arthritis already show that blockade of RAGE signalling by administration of soluble RAGE reduced clinical and histological evidence of arthritis. Besides administration of soluble RAGE in the affected joint, local gene therapy using a dominant negative RAGE is second option which can be explored. In addition, antagonistic effects of small molecules could be explored.

In *in vitro* studies of RA and OA pathology, often a single cell type is investigated; chondrocytes in OA and synoviocytes in RA. The results of this thesis however show that not only chondrocytes and synoviocytes can be activated by the same pathway, but also that the interaction between chondrocytes and synoviocytes can have an important role in cartilage degradation. Although it is believed that chondrocytes are the cells primarily involved in cartilage degradation in OA, OA synoviocytes can be activated *in vitro*. Therefore it is very likely that *in vivo* they not only play a role in thickening of the synovium, but can also influence cartilage degradation. This effect on cartilage degradation might be either directly by the production of MMPs able to cleave cartilage collagens, or by production of cytokines that can activate chondrocytes.
During RA, due to the pronounced inflammation, even more different cell types seem involved in the joint destruction. Synoviocytes are thought to be the cells primarily involved in cartilage degradation during RA. The ability of the synoviocytes to actually degrade the cartilage however seems to be dependent on the presence of viable chondrocytes. In addition, cytokines produced by macrophages can have a highly activating effect on both chondrocyte and synoviocyte activation. In choosing an *in vitro* model that leaves out certain cells or elements of the joint, one should be aware that removal of the interactions can have limitations on the interpretation of the outcome.

Although cell interactions in the rheumatoid joint play an important role, the fact that synoviocytes from the lining layer of RA patients show an altered and aggressive phenotype cannot be denied. *In vitro*, the synoviocytes from RA patient show a higher activation state and the *in vitro* invasiveness of the synoviocytes correlates with the disease activity of the patients (23). In this thesis, it is described that the healthy lining layer has an epithelial-like function and has several morphological properties specific for epithelium. However, because of the absence of tight-junctions and because the lining layer of the synovium is not completely continuous, the synovial lining is epithelial-like rather a real epithelium. Supporting the observation that synoviocytes of the lining layer of RA patients have undergone a transition towards a more aggressive morphology, markers for mesenchymal/fibrotic cells have been found in the synovial lining from RA patients. In addition, synovial fluid from RA patients can induce increased expression of fibrotic

proteins in healthy synoviocytes. These proteins are associated with increased production of extracellular matrix and an altered phenotype, resulting in the fibrosis which is seen in some RA patients, and with the aggressive and motile behaviour displayed by RA synoviocytes. Acquisition of the altered behaviour could well be the cause of the migration of the pannus towards the cartilage and the following cartilage destruction.
BMP-7 has previously been described as a therapeutic target for kidney fibrosis because of its antagonistic effects on TGF-β signalling. Our results show that BMP-7 can inhibit TGF-β induced changes in healthy synoviocytes, but also changes induced by other factors in synovial fluid from RA patients. This indicates a novel therapeutic role for BMP-7 in the treatment of RA, by favouring the maintenance of a quiescent phenotype of the synoviocytes from the lining layer. The fact that BMP-7 can be used as target for treatment of RA is even more interesting when we consider the stimulating effects of BMP-7 on cartilage synthesis. It therefore might not only inhibit cartilage destruction by aggressive synoviocytes, but can also stimulate repair of the cartilage if it might be damaged.

To summarize, this thesis describes a role for RAGE activation in cartilage degradation on chondrocytes and synoviocytes following accumulation of its ligands. Secondly, our results points out that the choice of matrix, and the interaction between chondrocytes-synoviocytes and cytokines derived from macrophages play an important role in *in vitro* models for cartilage degradation. Finally, the results of this thesis show that an EMT/fibrotic-like alteration in phenotype of the synoviocytes from the lining layer can be induced by TGF-β and synovial fluid, and that this alteration can be inhibited by BMP-7.
In conclusion, the results of this thesis provide us with some interesting targets in the treatment of cartilage destruction in OA and RA.

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Nederlandse Samenvatting

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Gewrichten

Gewrichten zijn belangrijk voor het bewegen van het lichaam. In een gewricht komen de uiteinden van twee botten samen, waardoor het gewricht als een scharnier werkt. Om te zorgen dat de botuiteinden soepel over elkaar kunnen glijden zijn ze bekleed met een dun laagje kraakbeen. Kraakbeen is een glad weefsel dat als een soort spons werkt; tijdens belasting van het gewricht wordt er water uitgedrukt en tijdens het ontlasten van het gewricht wordt er weer water opgenomen. Dit zorgt ervoor dat de druk die op de botten komt te staan tijdens de belasting deels opgevangen wordt.
De botten zijn aan elkaar verbonden met pezen en een gewrichtskapsel dat het geheel afsluit. Het gewrichtskapsel is aan de binnenzijde bekleed met een dun laagje cellen die samen het synovium vormen. Dit synovium is verantwoordelijk voor de productie van het synoviale vocht, de vloeistof die in het gewricht zit. Het synoviale vocht bestaat naast water uit voedingstoffen voor de kraakbeencellen (chondrocyten) en een aantal stoffen die een smerende werking hebben.

Cellen uit het gewricht

Chondrocyten

De kraakbeencellen ofwel chondrocyten zijn verantwoordelijk voor de productie van het kraakbeen. Het kraakbeen bestaat uit verschillende componenten die het kraakbeen zijn sponsachtige eigenschappen bezorgen. Collageen eiwitten vormen een netwerk die het kraakbeen zijn vorm geven. Daarnaast bevat kraakbeen ook andere eiwitten, proteoglycanen, die binnen het collageen netwerk gevangen zitten. Zij zorgen voor een sterke negatieve lading van het kraakbeen. Deze negatieve lading zorgt ervoor dat water het kraakbeen ingetrokken wordt tijdens ontlasting van het gewricht. De productie van het kraakbeen door chondrocyten wordt beïnvloed door verschillende factoren. Deze factoren kunnen bijvoorbeeld verandering in belasting zijn of stoffen in het synoviale vocht die afkomstig zijn van andere cellen uit het gewricht of zelfs elders uit het lichaam.

Synoviocyten

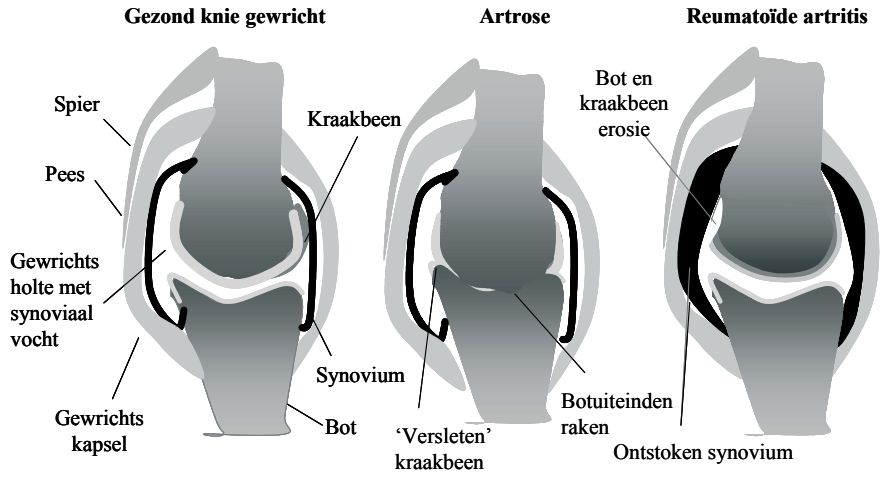
De binnenbekleding van de gewrichtskapsels heet het synovium en bestaat uit twee lagen. De laag aan de kant van het kapsel is de ‘sublining’, die bestaat uit losmazig weefsel met bloedvaten en relatief weinig cellen. De andere laag zit aan de kant van de gewrichtsholte en heet de ‘lining layer’. Deze lining layer is normaal 1 a 2 cellagen dik en bestaat uit twee celtypen; fibroblast-achtige cellen (synoviocyten) en macrofaag-achtige cellen.

woordenlijst	
synovium:	*bekleding van het gewrichtskapsel*
chondrocyten:	*kraakbeencellen verantwoordelijk voor het maken en onderhouden van het kraakbeen*
synoviocyten:	*fibroblast-achtige cellen uit het synovium, verantwoordelijk voor filtratie van synoviaal vocht uit bloed en productie van stoffen in het synoviaal vocht.*
AGEs:	*glyceringsproducten, ontstaan door spontane reactie van suikers met eiwitten (resulteert in verbinding tussen 2 eiwittten, of in suikerstaart aan eiwit).*
receptor:	*eiwit op membraan van cel die bepaalde andere eiwitten herkent en daarna een signaal doorgeeft aan de cel dat er iets moet gebeuren*
cytokines:	*eiwitten die door een cel gemaakt worden om te dienen als boodschapper voor andere cellen*

De synoviocyten zijn verantwoordelijk voor de filtratie van het synoviaal vocht uit bloed en de productie van de bovengenoemde stoffen met smerende werking. Daarnaast maken ze ook het weefsel aan waar ze in zelf liggen en kunnen ze eiwitten in het synoviaal vocht afscheiden. De macrofaag-achtige cellen zijn in staat om stoffen en andere cellen op te nemen en te verteren en zorgen zo voor afvoer van bijvoorbeeld bacteriën en eiwitten.

Gewrichtsaandoeningen

Reumatische aandoeningen zijn aandoeningen van het bewegingsapparaat. Reumatische ziekten zijn in drie verschillende groepen in te delen: weke-delen reuma, artrose en ontstekingsreuma. Bij weke-delen reuma zijn de spieren, kapsels, banden en pezen van het gewricht aangetast. Artrose is een slijtage ziekte, waarbij het verdwijnen van kraakbeen het belangrijkste kenmerk is. De meest voorkomende vorm van ontstekingsreuma is reumatoïde artritis (RA).



***Figuur 1**. Schematische weergave van een gezond kniegewricht vergeleken met een knie gewrichten van artorse en reuma patiënten.*

Artrose

Meer dan de helft van de mensen ouder dan 65 jaar heeft artrose in een of meerdere gewrichten. De klachten bij artrose zijn gewrichtspijn, bewegingsbeperking en soms zwelling van de aangetaste gewrichten. Tijdens artrose wordt in langzaam of snel tempo het kraakbeen aangetast en zal op den duur zelfs verdwijnen (figuur 1). Daarnaast ontstaan ook botvergroeiingen en een milde ontsteking (die voor de zwelling zorgt). Risicofactoren voor het krijgen van artrose zijn zowel genetische factoren als omgevingsfactoren. Een aantal factoren zijn overgewicht, vrouwelijk geslacht en trauma van het gewricht. De grootste risico factor lijkt veroudering van het kraakbeen te zijn.
Een van de processen die optreden tijdens veroudering is het ontstaan van niet-enzymatische glyceringsproducten (AGEs genoemd). AGEs worden gevormd tijdens de spontane reactie van suikers met eiwitten (zoals collagenen en proteoglycanen). AGEs kunnen in twee groepen gedeeld worden: degene die een eiwit-eiwit verbinding vormen (ofwel crosslinks) en degene die een aanhangsel aan eiwitten vormen (adducten).

Terwijl hun vorming continu doorgaat kunnen AGEs niet zomaar verwijderd worden, ze verdwijnen alleen als het eiwit waar ze aan verbonden zijn verdwijnt. Hierdoor hopen de AGEs op in eiwitten met een lage vernieuwingssnelheid, zoals de collageen eiwitten in kraakbeen. Door de ophoping van AGEs in kraakbeen veranderen de mechanische eigenschappen van het kraakbeen. Daarnaast hebben chondrocyten eiwitten op de membraan (receptoren) die de AGEs herkennen. Door binding van de AGEs aan deze receptoren kunnen verschillende processen in de chondrocyten worden aangezet.

Reumatoïde artritis (RA)

RA komt bij ongeveer 1% van de bevolking in de westerse wereld voor. Het is een ziekte die meer voorkomt bij vrouwen dan bij mannen. Hoewel bij RA net als bij artrose de precieze oorzaken niet bekend zijn, spelen genetische factoren mogelijk een rol. In tegenstelling tot artrose is RA een auto-immuunziekte, waarbij de ontsteking van het synovium het belangrijkste kenmerk van de ziekte is. De ontsteking van het synovium wordt gevolgd door de aantasting van het kraakbeen. Naast de gewrichten kunnen bij RA ook andere organen, zoals hart en bloedvaten, longen en huid, aangetast worden. Daarnaast worden bij RA in het bloed antistoffen aangetroffen tegen lichaamseigen eiwitten.

Tijdens de ontsteking van het synovium die optreedt in RA is er een toename van immuuncellen in de sublining en verdikking van de lining layer. De lining layer kan tijdens RA uit wel 10 cel lagen bestaan (figuur 1). De synoviocyten die verantwoordelijk lijken te zijn voor de verdikking van de lining layer worden ook gevonden op de plaatsen waar het kraakbeen verdwijnt. Daardoor wordt aangenomen dat zij een belangrijke rol spelen in de afbraak van kraakbeen tijdens RA.

Vaak is er bij RA sprake van een overmatige productie van synoviaal vocht (de gewrichtsvloeistof), wat bijdraagt aan de zwelling van de gewrichten. Door de verandering in samenstelling van het synovium verandert echter ook de samenstelling van het synoviale vocht. Verschillende cytokines (eiwitten die door cellen gemaakt worden als boodschapper voor andere cellen) komen in verhoogde mate voor in het synoviaal vocht van RA patiënten. Een aantal van deze cytokines zijn stoffen die ook kunnen binden aan de receptor voor glycerings producten (zie onder).

De receptor voor glycerings producten (RAGE)

Cellen kunnen communiceren met hun omgeving door middel van receptoren. Receptoren zijn eiwitten die bepaalde stoffen herkennen en daarna een signaal aan de cel doorgeven. Niet alle cellen hebben alle receptoren, daardoor reageert het ene celtype wel op een stof, terwijl een ander celtype dat misschien niet doet.

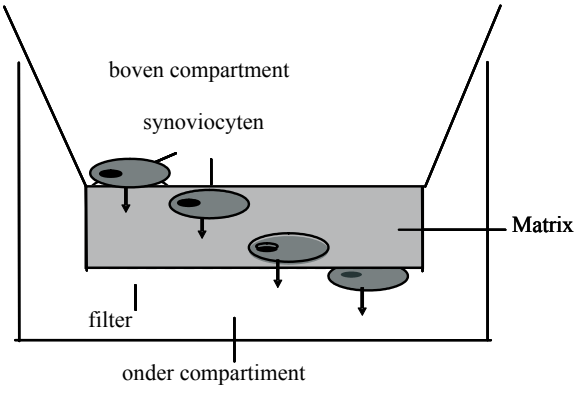
RAGE is een receptor die voorkomt op het oppervlak verschillende cellen. Herkenning van een aantal stoffen, waaronder glyceringsproducten (genaamd AGEs), leidt tot activatie van de cellen. Naast glycerings producten worden ook andere eiwitten herkend door RAGE, waarvan de concentraties in verschillende ziektes, zoals RA, kanker en fibrose, verhoogd zijn.

In het gewricht hebben zowel immuuncellen, synoviocyten en chondrocyten RAGE op hun oppervlak. Daardoor zijn ze in staat om te reageren op de AGEs die verhoogd voorkomen in kraakbeen van artrose patiënten en op de verschillende liganden die verhoogd zijn in het synoviaal vocht van RA patiënten. In dit proefschrift is bestudeerd

hoe activatie van RAGE op synoviocyten en chondrocyten een rol speelt bij kraakbeenafbraak tijdens artrose en RA.

Resultaten van dit proefschrift

Het invasiemodel dat meestal gebruikt voor het bestuderen van synoviocyt invasie en activatie is niet optimaal. De matrix die hiervoor normaal gesproken gebruikt wordt bestaat uit een ander type collageen dan in kraakbeen voorkomt. Daarnaast zijn in kraakbeen nog een heel aantal andere eiwitten aanwezig. Bij afbraak van verschillende soorten matrix zijn verschillende enzymen betrokken. Zo is bijvoorbeeld een van de enzymen die het collageen uit het invasie model kan afbreken niet in staat het collageen uit kraakbeen af te breken. Daarom is het belangrijk om in een model voor kraakbeen afbraak ook daadwerkelijk de componenten van kraakbeen te betrekken. Er zijn ook aanwijzingen dat de interactie tussen synoviocyten en chondrocyten belangrijk is bij kraakbeenafbraak. Daarom is het belangrijk om behalve synoviocyten ook chondrocyten in het invasie model te betrekken. In ***hoofdstuk 2*** wordt een model beschreven waarbij chondrocyten uit kraakbeen van koeienpoten worden geïsoleerd en daarna gebruikt worden om een kraakbeen-achtig schijfje te maken. De cellen maken zelf nieuw kraakbeen, dat qua samenstelling heel veel lijkt op het normale kraakbeen. Dit nieuw gevormde kraakbeen-achtige schijfje met daarin chondrocyten vervangt de matrix uit het invasie model uit figuur 2. Op het kraakbeen-achtige schijfje worden synoviocyten gezet. Als uitleesmaat wordt de afbraak van de matrix gemeten in plaats van doorgroei van synoviocyten. Het voordeel hiervan is dat zo ook de kraakbeenafbraak door de chondrocyten zelf wordt gemeten. Met dit nieuwe model is de interactie bestudeerd tussen synoviocyten en chondrocyten, alsmede het effect van een aantal eiwitten gemaakt door macrofagen. De resultaten van de studie laten zien dat synoviocyten levende chondrocyten nodig hebben om kraakbeenafbraak te induceren. Daarnaast hebben de gebruikte eiwitten (cytokines IL-1β en TNF-α) van de macrofagen alleen een effect op de chondrocyten in dit model. Dit leidt tot de conclusie dat hoewel synoviocyten een rol lijken te spelen in de kraakbeenafbraak in RA, de rol van chondrocyten en de interactie tussen synoviocyten en chondrocyten ook erg belangrijk is.



Figuur 2. *Het invasiemodel voor synoviocyten. Synoviocyten worden in het boven compartiment boven op een matrix uitgezaaid. Na drie dagen zal een deel van de cellen door de matrix en het filter heen zijn gemigreerd. Als ze de onderkant van het filter bereiken, hechten ze daaraan vast. Daar kunnen de cellen dan geteld worden, als maat voor het invasieve gedrag.*

Om de rol van RAGE activatie in artrose te bestuderen zijn chondrocyten en synoviocyten gestimuleerd met AGEs, die zij in artrotisch kraakbeen of in het synoviale vocht tegen kunnen komen. De resultaten, beschreven in ***hoofdstuk 3***, laten zien dat chondrocyten hun eigen kraakbeen gaan afbreken na contact met AGEs. Ze gaan meer enzymen maken die het kraakbeen kunnen afbreken en daarnaast wordt de nieuwe productie van kraakbeen minder. Synoviocyten van artrose patiënten die in contact komen met AGEs kunnen sneller ingroeien door een invasie systeem dat als model dient voor de afbraak en invasie van kraakbeen (zie figuur 2). Daarnaast is gevonden dat ook synoviocyten meer enzymen gaan maken die kunnen leiden tot afbraak van kraakbeen.

Eiwitten worden gecodeerd in het genetische materiaal dat in iedere cel aanwezig is. Het genetische materiaal verschilt per persoon. Sommige genen (een gen is een stuk DNA dat codeert voor een bepaald eiwit) kunnen in verschillende vormen voorkomen. Sommige vormen komen vaak voor binnen een populatie, andere vormen komen niet zo vaak voor. Andere genen hebben normaal maar één vorm. Als er dan wel een andere vorm gevonden wordt, die in een klein gedeelte van de mensen voorkomt, noemen we dat een mutatie. In het RAGE gen zijn ook mutaties gevonden. Sommige van deze mutaties hebben geen functie: het RAGE gen verandert niet zichtbaar van functie. Er is echter ook een mutatie, die RAGE G82S genoemd wordt, die wel leidt tot een verandering in functie. Door dr. Hoffman en haar collega's is gevonden dat deze mutatie verhoogd voorkomt in patiënten met RA. Na correctie voor een factor waarvan al bekend is dat het bij RA betrokken is verdwijnt het verband echter. Omdat de groep van Hoffman slechts een relatief kleine groep mensen heeft bestudeerd en de mutatie in niet zoveel mensen voorkomt, kan het zijn dat de kleine aantallen zorgen dat het gevonden verband niet statistisch significant is. De resultaten van een soortgelijk onderzoek in een groep RA patiënten van het LUMC worden beschreven in ***hoofdstuk 4***. Om uit te sluiten dat het verband tussen de mutatie en RA verdwijnt doordat de aantallen te klein worden hebben we dit onderzoek uitgevoerd in een veel grotere groep patiënten en controles. Uit ons onderzoek blijkt dat de RAGE mutatie inderdaad niet onafhankelijk van de andere factor met RA associeert.
De afwezigheid van een associatie tussen de mutatie van het RAGE gen en RA wil niet zeggen dat RAGE geen rol kan spelen in RA. Zoals boven beschreven zijn meerdere eiwitten die door RAGE herkend worden verhoogd aanwezig in RA. Om het effect van deze eiwitten op het gedrag van synoviocyten van RA patiënten te bestuderen, is het invasie model gebruikt dat beschreven is in figuur 2 (***hoofdstuk 5***). Toevoeging van twee eiwitten die door RAGE herkend wordt, leidt tot verhoogde invasie. Als we daarbij een antilichaam tegen RAGE toevoegen, dat zorgt dat RAGE niet meer geactiveerd kan worden, dan wordt de invasie niet verhoogd. Hieruit kunnen we concluderen dat RAGE een belangrijke rol kan spelen bij de activatie van synoviocyten in RA.

Tot slot van het onderzoek is gekeken naar de veranderingen die de synoviocyten van RA patiënten ondergaan. De synoviale lining layer van gezonde mensen heeft min of meer dezelfde functie als epitheelcellen. Epitheelcellen zijn de cellen die de buitenste laag vormen van bijvoorbeeld huid, darmwand of klieren; ze vormen de scheiding tussen 'binnen' en 'buiten'. Zij hebben als functie om een barrière te vormen tussen de buitenwereld of de holte die ze omsluiten en het onderliggende weefsel. Daarnaast zijn de epitheelcellen verantwoordelijk voor de uitwisseling van stoffen en eventueel productie van andere stoffen. Epitheelcellen liggen erg dicht tegen elkaar aan en hebben

verschillende soorten verbindingen waarvan sommige ervoor zorgen dat er niet zomaar stoffen tussen de cellen door kunnen gaan en andere juist verantwoordelijk zijn voor communicatie en uitwisseling van stoffen tussen de cellen. In ***hoofstuk 6*** wordt beschreven dat de synoviale lining layer van gezonde mensen een aantal van eigenschappen bevat van epitheel cellen. Deze eigenschappen zijn ook gevonden in patiënten met RA. In patiënten met RA zijn echter ook eigenschappen gevonden van cellen die normaal in fibreus (litteken) weefsel voorkomen.
In fibrose is sprake van abnormale wondheling. De betrokken cellen gaan meer delen en meer collageen maken, waardoor het littekenweefsel gevormd wordt. In RA patiënten zien we dat de lining layer, die normaal maar uit 1 of 2 cellagen bestaat, uit meerdere cellagen is opgebouwd en dat er ook extra collageen wordt geproduceerd. Daarnaast is ook een molecuul gevonden dat specifiek is voor de cellen die verantwoordelijk zijn voor fibrose en dat ook voorkomt in kankercellen (die net als de cellen van RA patiënten zich kunnen verplaatsen en ander weefsel kunnen afbreken). Om te bestuderen of het synoviale vocht van RA patiënten deze verandering kan veroorzaken, zijn synoviocyten van gezonde mensen gekweekt met synoviaal vocht van RA patiënten. Daarbij zagen we dat de cellen meer fibrotische eigenschappen kregen.
Deze veranderingen treden ook op bij fibrose van de nier, en zijn te remmen door een stof genaamd BMP-7, waardoor de nierfibrose afneemt. Normaal speelt deze stof een rol bij de ontwikkeling van bot en kraakbeen, maar heeft dus ook andere (anti-fibrotische) eigenschappen. Toevoeging van BMP-7 aan de synoviocyten van gezonde patiënten die met synoviaal vocht gestimuleerd worden leidt ertoe dat de fibrotische veranderingen onderdrukt worden; de cellen behouden meer van hun gezonde karakter. Niet allen is BMP-7 in staat om de veranderingen in synoviocyten te onderdrukken, het is ook in staat om kraakbeen aanmaak te stimuleren. Dit betekent dat BMP-7 therapie een interessante behandelingsmethode zou kunnen zijn voor RA patiënten.

Appendix

Figures with chapter 6 – EMT in rheumatoid arthritis

Figure 1. HE staining on healthy and arthritic synovial tissue. HE staining was performed on synovial biopsies healthy subjects (**A**) and RA patients (**B**). Arrows indicate synovial lining.

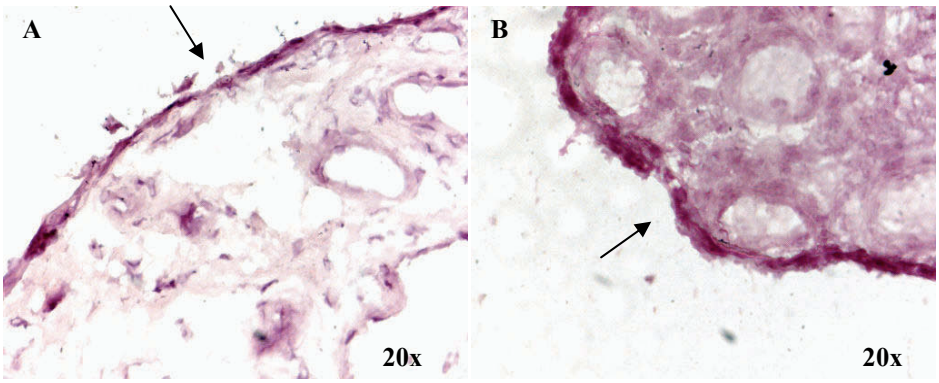


Figure 2. E-cadherin staining in healthy and arthritic synovial tissue. Synovial biopsies from healthy human subjects (**A**) and RA patients (**C**) showed a positive E-cadherin staining in the lining layer. Relative isotype controls are shown in **B** and **D**. Arrows indicate E-cadherin positive cells from the lining layer.

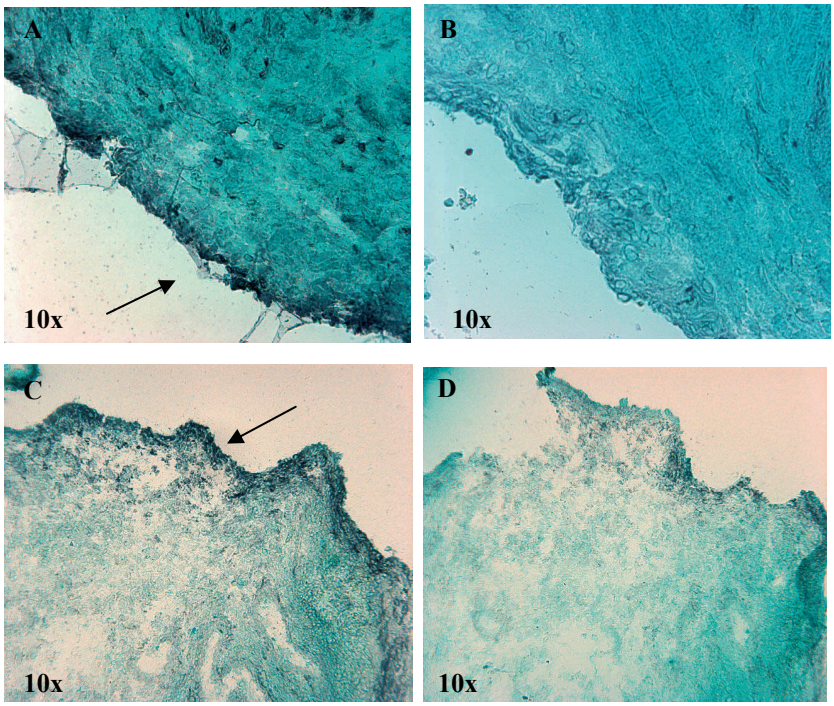


Figure 3 A-H. Collagen type IV staining in healthy and arthritic synovial tissue. Type IV collagen was found in the lining layer of synovial biopsies from healthy human subjects (**A**) and RA patients (**C**). Relative isotype controls are shown in **B** and **D**. Double staining was performed in order to reveal the cellular origin of the type IV collagen. Double staining was found in the lining layer (**E**). For a clearer picture, succeeding slices were stained with CD55 (**F**) or type IV collagen (**G**). Isotype control is shown in figure **H**. Arrows indicate type IV collagen, CD55 or double staining in the lining layer.

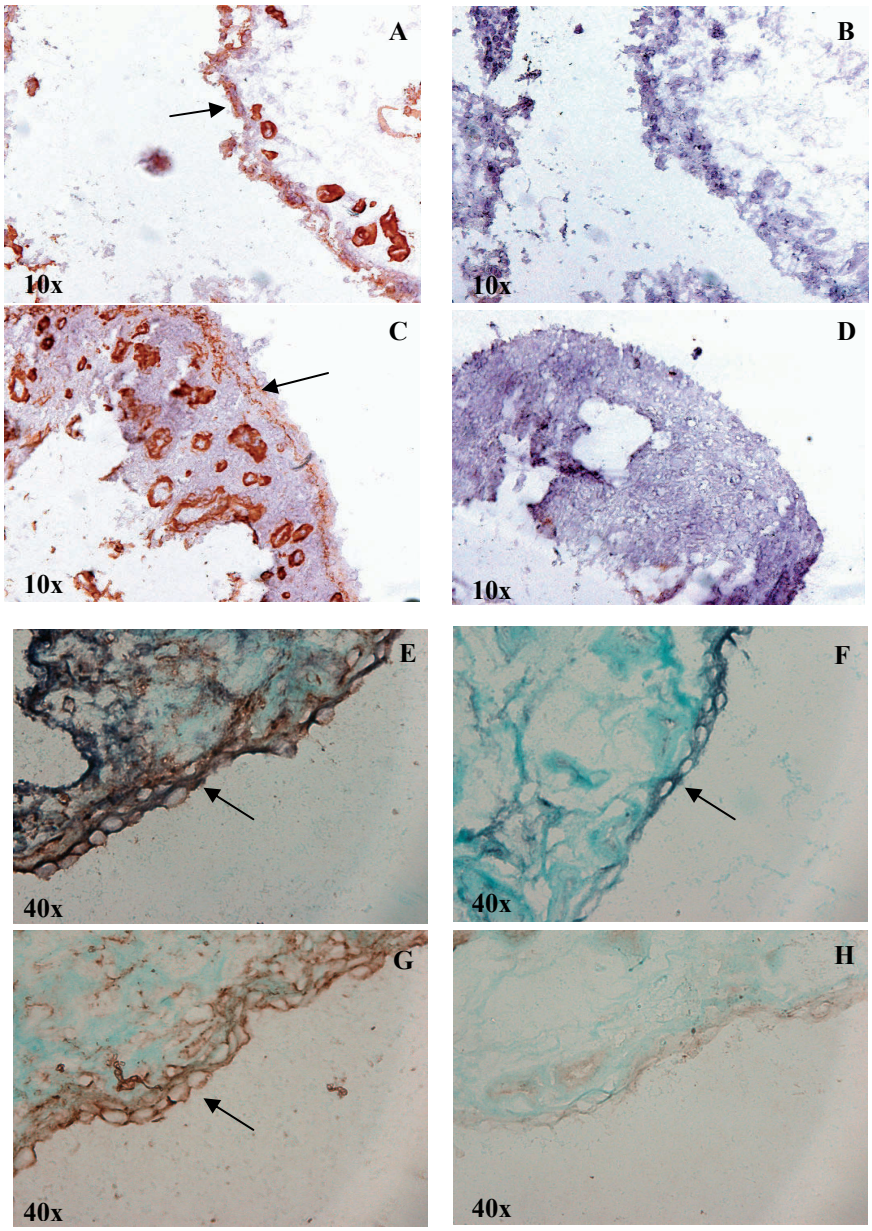
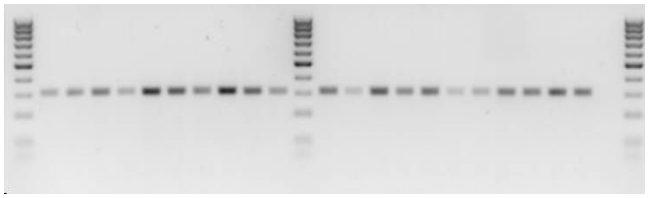
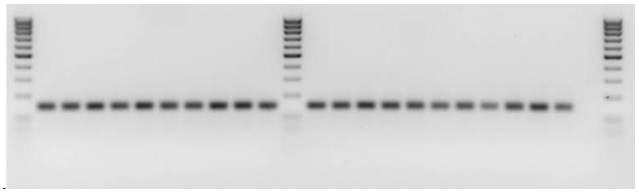


Figure 3 I. PCR results are shown in figure 3I. **Upper** picture shows type IV collagen mRNA expression in FLS from 10 RA patients and 11 healthy controls. **Lower** picture shows β_2M mRNA expression in the same samples.

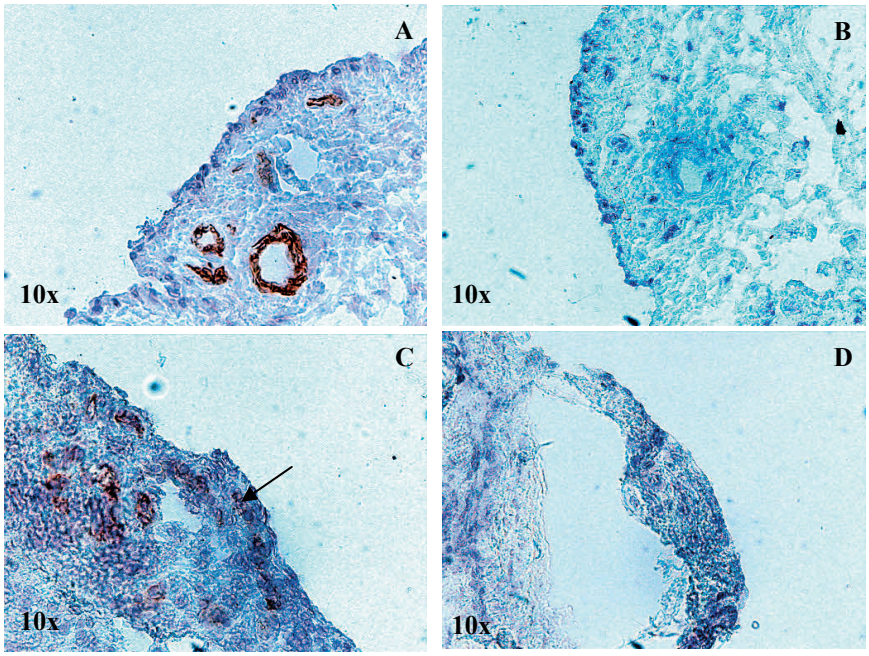


Type IV collagen



β_2M

Figure 4. α-sma staining in healthy and arthritic synovial tissue. No α-sma staining was found in the lining layer of healthy synovial tissue (**A**). In synovial biopsies RA patients, cells positive for α-sma expression were found in the lining layer (**C**). Relative isotype controls are shown in **B** and **D**. Arrows indicate α-sma positive cells from the lining layer.



List of abbreviations

AGE	**A**dvanced **G**lycation **E**ndproduct
RAGE	**R**eceptor for **AGE**s
HMGB	**H**igh **M**obility **G**roup **B**ox protein
GAG	**G**lycos**A**mino**G**lycan
OA	**O**steo**A**rthritis
RA	**R**heumatoid **A**rthritis
FLS	**F**ibroblast-**L**ike **S**ynoviocyte
SF	**S**ynovial **F**luid
PBS	**P**hosphate **B**uffered **S**aline
BSA	**B**ovine **S**erum **A**lbumin
MMP	**M**atrix **M**etallo**P**roteinase
TIMP	**T**issue **I**nhibitor of **MMP**
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
HP	**H**ydroxylyxyl**P**yridinoline
LP	**L**ysyl**P**yridinoline
IL	**I**nter**L**eukin
FCS	**F**oetal **C**alf **S**erum
ELISA	**E**nzym **L**inked **I**mmunosorbentAssay
ECM	**E**xtra**C**ellular **M**atrix
GM-CSF	**G**ranulocyte and **M**onocyte **C**olony **S**timulating **F**actor
DNA	**D**eoxyribo**N**ucleic **A**cid
CIA	**C**ollagen **I**nduced **A**rthritis
BMP	**B**one **M**orphogenetic **P**rotein
HLA	**H**uman **L**eukocyte **A**ntigen
HS	**H**uman **S**erum
ICAM	**I**ntercellular **A**dhesion **M**olecule
IMDM	**I**scove's **M**odified **D**ulbecco's **M**edium
DMEM	**D**ulbecco's **M**odified **E**agle's **M**edium
MCP	**M**onocyte **C**hemoattractant **P**rotein
TNF	**T**umor **N**ecrosis **F**actor
SCID	**S**evere **C**ombined **I**mmunodeficiency **D**isorder
TGF	**T**ransforming **G**rowth **F**actor
α-SMA	**alpha S**mooth **M**uscle **A**ctin
TLH	**T**elopeptide **L**ysyl **H**ydroxylase
COL1A2	**Col**lagen type **I α2**
β_2M	**β-2-m**icroglobulin
GAPDH	**G**lycer**A**ldehyde 3-**p**hosphate **DeH**ydrogenase

mRNA	**m**essenger **RNA**
lys	**Lys**ine
pro	**Pro**line
MHC	**M**ajor **H**istocompatibility **C**omplex
COMP	**C**artilage **O**ligomeric **P**rotein
ADAM	**A D**isintegrin **A**nd **M**etalloproteinase
ADAMTS	**ADAM** with **T**rombospondin motif**s**
MT-MMP	**M**embrane **T**ype **MMP**

List of publications

1) **Marjan M.C. Steenvoorden**, Tom W.J. Huizinga, Nicole Verzijl, Ruud A. Bank, H. Karel Ronday, Hilco A.F. Luning, Floris P.J.G. Lafeber, René E.M. Toes, Jeroen DeGroot. Activation of Receptor for Advanced Glycation End Products in Osteoarthritis Leads to Increased Stimulation of Chondrocytes and Synoviocytes. *Arthritis and Rheumatism (2006) 54 (1): 253-263*

2) **M.M.C. Steenvoorden**, A.H.M. van der Helm-van Mil, G. Stoeken, R.A. Bank, R.R.P. DeVries, T.W.J. Huizinga, J. DeGroot, R.E.M. Toes. The RAGE G82S Polymorphism Is Not Associated with Rheumatoid Arthritis Independent of HLA-DRB1*0401. *Rheumatology(2006) 45 (4): 488-490.*

3) **Marjan M.C. Steenvoorden**, Ruud A. Bank, H, Karel Ronday, René E.M. Toes, Tom W.J. Huizinga, Jeroen DeGroot. Fibroblast-like Synoviocyte-Chondrocyte Interaction in Cartilage Degradation. *Clinical and Experimental Rheumatology -in press.*

4) **Marjan M.C. Steenvoorden**, René E.M. Toes, H. Karel Ronday, Tom W.J. Huizinga, Jeroen DeGroot. RAGE activation induces invasiveness of RA FLS. *Submitted for publication.*

5) **Marjan M.C. Steenvoorden**, Tanja C.A. Tolboom, Gabri van der Pluijm, Clemens Löwik, Cornelis P.J. Visser, Jeroen DeGroot, Adriana C. Gittenberger-DeGroot, Marco C. DeRuiter, Bert J. Wisse, Tom W.J. Huizinga, René E.M. Toes. Transition of healthy to diseased synovial tissue in RA is associated with gain of mesenchymal/fibrotic characteristics. *Arthritis Research & Therapy –in press.*

Nawoord

Het werken aan dit proefschrift is niet alleen heel leerzaam geweest, maar ook erg gezellig. Het werken op twee afdelingen was voor mij een groot plezier. Niet alleen kreeg ik nuttige tips van mensen met verschillende benaderingen, maar ook heb ik tijdens en tussen de proeven genoten van het gezelschap van een dubbel team van collega's.

Zoals ieder proefschrift is ook deze tot stand gekomen met de hulp van veel mensen. Ik wil iedereen die mij met raad en daad heeft bijgestaan van harte bedanken!
Frits, Nico en Geesje, bedankt voor alle hulp bij de HPLC en natuurlijk de soms wat (te) grote kweekexperimenten, door jullie hulp bleef het allemaal overzichtelijk. Benno, bedankt voor de raad bij het isoleren en kweken van de synoviocyten en Karel voor het aanleveren van het synovium weefsel: het is een heel mooie voorraad geworden, waarvan ik heel goed gebruikt heb kunnen maken.
Elly, Anne-Marie, Annemarie en Joline wil ik bedanken voor de introductie in het eiwit en RNA- real time werk. Bep en Nivine hebben mij een introductie in de immunohistochemie gegeven en later input gegeven bij brainstormen als het even niet liep. Voor het RAGE-genetica artikel wil ik Gerrie bedanken voor haar hulp bij het typeren van het RAGE polymorfisme bij de EAC patiënten en Fina en Annette voor hun hulp bij de statistische kant. Yvonne en Tanja: zowel in Leiden als in het buitenland op congres waren de gezellige AIO gesprekken erg leerzaam en leuk.

Naast deze mensen zijn er natuurlijk ook alle coauteurs die mij geholpen hebben met alle hoofdstukken en de mensen die niet genoemd zijn maar weten wat ze hebben bijgedragen. Ook zij allen bedankt!

Curriculum Vitae

Marjan Steenvoorden werd geboren op 12 april 1979 te Noordwijk. Na het behalen van haar atheneum diploma op de openbare scholengemeenschap Northgo in 1997 begon ze hetzelfde jaar aan de studie Biologie aan de Universiteit Leiden. In het derde jaar van de studie heeft zij de richting Medische Biologie gevolgd. Vervolgens heeft zij in het derde en vierde jaar tijdens haar stage bij de afdeling Maag-, Darm- en Leverziekten in het Leids Universitair Medisch Centrum onder leiding van dr. Masclee onderzoek gedaan naar de intrinsieke reflexen van het colon bij patiënten met het prikkelbare darm syndroom. Dit onderzoek werd beloond met de prijs voor 'het beste door een student uitgevoerde onderzoek' op de bijeenkomst voor maag- darm-, lever artsen. De opleiding werd afgesloten met haar afstudeerstage bij de afdeling Endocrinologie onder leiding van dr M. Karperien, waar zij onderzoek heeft gedaan naar modellen voor kraakbeenontwikkeling.
Na haar afstuderen als doctorandus november 2001 is zij in mei 2002 begonnen aan haar promotieonderzoek bij de afdeling Reumatologie van het LUMC en de afdeling Biomedical Research van TNO kwaliteit van leven. Dit onderzoek werd begeleid door Professor Dr. TWJ Huizinga en Dr. REM Toes verbonden aan de afdeling Reumatologie van het LUMC en door Dr. J De Groot van de afdeling Biomedical Research van het TNO. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Na haar promotie hoopt de auteur aan de slag te gaan als postdoc.