UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Receptor for Advanced Glycation Endproducts and S100A11 Modulate Pathologic Chondrocyte Differentiation and Dysregulated Cartilage Matrix Catabolism in Osteoarthritis

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

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EPIGRAPH

"Experience comes from what we have done.

Wisdom comes from what we have done badly."

-Theodore Levitt

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LIST OF ABBREVIATIONS

ACL-T	Anterior cruciate ligament tear
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motif
AGER	Advanced glycation endproducts receptor
ATRA	All-*trans* retinoic acid
BMP	Bone morphogenetic protein
Ca^{2+}	Calcium
CXCL1	Growth related oncogene alpha
CXCL8	Interlukin-8
DMOAD	Disease-modifying OA drug
DMM	Destabilization of medial meniscus
DN	Dominant negative
ECM	Extracellular matrix
ERK	Extracellular signal-related kinase
GAG	Glycosaminoglycans
GTP	Guanidine triphosphate
HMGB1	High mobility group box 1
ICE	IL-1 converting enzyme
IL-1β	Interlukin-1 beta
IL-1ra	Interlukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
JNK	Jun N-termianl kinase

KO	Knockout
K3R/Q102N	S100A11 transamidation site mutant
MAPK	Mitogen activated protein kinase
MKK3	Mitogen activated protein kinase kinase 3
MMP-13	Matrix metalloproteinase 13
MRI	Magnetic resonance imaging
NF-κB	Nuclear factor-kappa B
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OPG	Osteoprotogerin
OPN	Osteopontin
PG	Proteoglycans
PM	Partial meniscectomy
RAGE	Receptor for advanced glycation endproducts
RANKL	Receptor activator of NFκB ligand
RUNX2	Runt related gene 2
sRAGE	soluble RAGE
TACE	TNFα converting enzyme
TG2	Transglutaminase 2
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
WT	wild type

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Transforms S100A11 Calgranulin into a Pro-Catabolic Cytokine for Chondrocytes", 180(12):8378-85, 2008. I was the primary researcher and author and the co-author listed in this publication directed and supervised the research which forms the basis of this chapter.

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ABSTRACT OF THE DISSERTATION

The Receptor for Advanced Glycation Endproducts and S100A11 Modulate Pathologic Chondrocyte Differentiation and Dysregulated Cartilage Matrix Catabolism in Osteoarthritis

by

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Changes within the synovium and cartilage associated with low-grade inflammation modulate the pathogenesis of OA, and many studies have shown that the inflammatory cytokines/chemokines IL-1β, TNFα, CXCL1 and CXCL8 significantly contribute to the disease progression. The Receptor for Advanced Glycation Endproducts (RAGE) and S100/calgranulins have been implicated in the pathogenesis of chronic arterial, renal, and neurological degenerative states associated with low-grade tissue inflammation. Therefore, the studies in this dissertation proposed that the association of RAGE and its ligands (specifically S100A11) are important in the pathogenesis of OA.

RAGE and S100A11 expression were upregulated in OA cartilage compared to normal cartilage. CXCL8-, TNFα-, and S100A11-induced chondrocyte hypertrophy were suppressed by treatment with soluble RAGE (sRAGE) or RAGE-specific blocking

antibodies. Finally, it was determined that S100A11 induced MKK3 and p38 MAPK activation.

S100/calgranulins normally exist as homo/heterodimers and the type of bond formed and subsequent conformation affect their activities. As it was previously determined that S100A11 was a substrate for Transglutaminase 2 (TG2), an S100A11 transamidation mutant was created (S100A11 K3R/Q102N), which formed only monomers. In mouse cartilage explants and chondrocytes, S100A11 K3R/Q102N mutant lost the capacity to signal via the p38 MAPK pathway or induce chondrocyte hypertrophy and glycosaminoglycans (GAG) release. S100A11 failed to induce hypertrophy and GAG release in $RAGE^{-/-}$ and $TG2^{-/-}$ cartilages.

The role of RAGE in the pathogenesis of OA was examined *in vivo*. Instability was induced surgically on $RAGE^{-/-}$ and congenic wild-type controls with an anterior cruciate ligament tear (ACL-T) through a blind "stab" incision. Cartilage degeneration, osteophyte formation and type X collagen expression significantly increased as instability increased, but there was no significant difference between $RAGE^{-/-}$ and congenic wild-type controls. Though RAGE gene deletion does not protect cartilage from degeneration in the ACL-T model of OA, the function of RAGE has not been assessed in another model of surgically-induced OA. In addition, the role of RAGE ligands, involvement of other receptors that recognize RAGE ligands and sRAGE have not been addressed.

Taken together, the studies in this dissertation demonstrate critical roles for RAGE and S100A11 in modulating pathologic chondrocyte hypertrophic differentiation and dysregulated matrix catabolism.

CHAPTER 1

Introduction

1.1 Cartilage

There are three types of cartilage present in the human body: fibrocartilage (e.g. invertebral disks), elastic cartilage (e.g. larynx and external ear), and hyaline cartilage (e.g. articular surfaces of synovial joints) [1].

Hyaline cartilage matrix is composed of a highly regulated network of types II, IX and XI collagen fibrils which interact with proteoglycans-glycosaminoglycans aggregates (primarily aggrecan-hyaluronan) (Figure 1.1). This interaction is sustained by electrostatic contact and adhesive glycoproteins including tenascin and fibronectin. Other proteoglycan molecules such as decorin, biglycan and fibromodulin help stabilize the matrix [1].

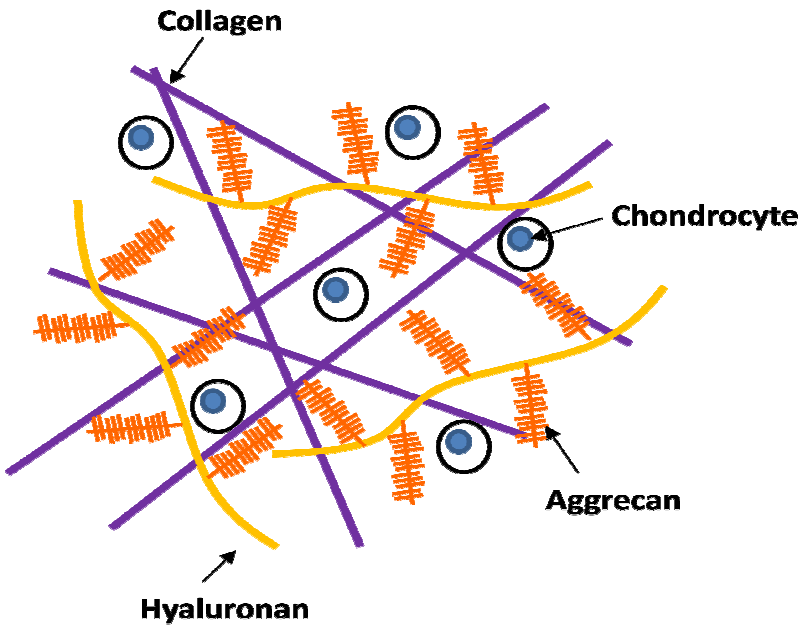


FIGURE 1.1
Diagram of the major components of the cartilage matrix. Articular cartilage is comprised of chondrocytes surrounded by a matrix of collagens (blue line), and proteoglycans-glycosaminoglycans aggregates (red "bottle brush" and yellow line).

Aggrecan has a high negative charge due to the attachment of chondroitin and keratan sulfate chains to the core protein. This negative charge draws water into the cartilage matrix collectively providing tensile strength and compressive resistance, crucial in distributing load in weight-bearing joints [2].

Mature articular cartilage is a heterogeneous tissue with four distinct regions: the superficial zone, middle zone, deep zone, and the calcified zone. The cell density, collagen and water content progressively decreases from superficial zone to deep zone [3, 4]. The calcified zone is formed as a result of endochondral ossification and offers mechanical support between the uncalcified cartilage and the subchondral bone [5].

Mature adult chondrocytes are the only cellular component of articular cartilage and are fully differentiated. These cells comprise 2-5% of the tissue volume and are left over from the resting, proliferating and prehypertrophic chondrocytes that developed the original cartilage matrix during chondrogenesis and growth plate formation [2].

Due to the lack of a vascular supply and innervation in the tissue, the chondrocytes are virtually inactive metabolically, but can respond to mechanical stimuli, growth factors and cytokines that influence homeostasis. In normal adult articular cartilage the chondrocytes synthesize matrix components very slowly. The half life of collagen turnover is 100 years and aggrecan turnover is between 3-24 years [6-8].

1.2 Osteoarthritis

1.2.1 Pathogenesis

Osteoarthritis (OA), the most common form of arthritis, is a degenerative joint disease characterized by progressive destruction of articular cartilage, loss of joint space, subchondral bone sclerosis and presence of bony outgrowths (osteophytes) [2]. Additionally, biochemical and morphological alterations of the synovial membrane, joint capsule, ligaments and periarticular muscles is often associated with disease progression. OA can occur in any joint, but it is most commonly develops in the hand, knee, hip and foot [9]. Clinical presentations of OA include use-related pain, stiffness, tenderness, and decreased range of motion [10].

The etiology, pathogenesis and progression of OA are incompletely understood. This understanding is confounded by poor epidemiological studies, the failure to detect early disease and the lack of association between OA symptoms and radiographic findings [11]. It was once thought OA is a result of normal "wear and tear" from aging cartilage. Now it is believed OA is an active process whereby the chondrocyte fails to maintain the balance between anabolism and catabolism, leading to the characteristic cartilage thinning [12].

In early OA, there is an increased synthesis of cartilage matrix as an initial attempt at repair and the chondrocytes proliferate to form clones or clusters. The synthesis and secretion of matrix degrading enzymes from the chondrocytes and synoviocytes increases as OA progresses leading to the weakening of the proteoglycans (PG) and collagen network. Despite the attempt at early repair, the limited matrix synthesis is overwhelmed by the increased activity of matrix degrading enzymes. The

irregular, fibrillated cartilage surface, characteristic of late OA, is irreparable since adult articular chondrocytes cannot regenerate the normal cartilage matrix. Eventually severe cartilage ulcerations expose the underlying bone [13].

Synovial fluid and cartilage from OA patients exhibit increased matrix metalloproteinases (MMPs) which are localized to regions of cartilage degradation [14-17]. Collagenases (MMP-1, -8, and -13) and stromelysin (MMP-3) specifically degrade collagens and proteoglycans. A major role for MMP-13 in cartilage degradation has been suggested since it preferentially degrades type II collagen [18]. MMP-2 and -9, cathepsins B, L, and D, and plasminogen activators are also produced by chondrocytes and may degrade different matrix components or participate in proteinase activation [2]. MMP-3, -13 and -8 have the potential to degrade aggrecan [19-21], but it is more effectively degraded by the aggrecanases ADAMTS-4 and -5 [22].

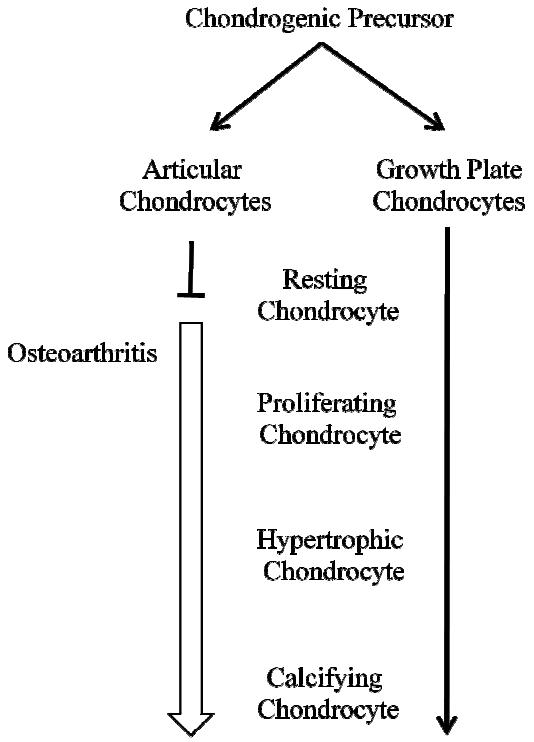


FIGURE 1.2
Stages of chondrocyte maturation. The differentiation and maturation of growth plate chondrocytes is highly regulated resulting in endochondral ossification. Articular chondrocytes, which derive from a similar chondrogenic precursor, are normally halted in the resting stage. However, when OA occurs, articular chondrocytes begin to differentiate similar to growth plate chondrocytes eventually leading to calcification.

Articular chondrocytes in OA undergo hypertrophic differentiation, partially recapitulating the chondrocyte life-cycle in endochondral ossification (Figure 1.2) [23]. In physiologic endochondral ossification, the maturation of chondrocytes from resting cells to hypertrophic cells is critical to the restructuring of the matrix eventually resulting in calcification and bone formation [24, 25]. The pathologic maturation of chondrocytes to hypertrophy in OA articular cartilage also promotes matrix calcification [26]. Chondrocyte hypertrophy in OA is associated with aberrant type X collagen expression and can promote dysregulation of matrix repair by increased expression of MMPs,

alkaline phosphatase (AP), parathyroid hormone related peptide (PTHrP), Indian Hedgehog (Ihh) and RUNX-2 transcription factor [27, 28].

1.2.2 Inflammatory Mediators

As there are few neutrophils in the synovial fluid and a lack of systemic inflammation, OA is not considered to be classically inflammatory [29]. Nevertheless, there is evidence of a low-grade inflammatory state associated with classic inflammatory mediators such as cytokines, chemokines, prostaglandins (PGE) and nitric oxide (NO) [30, 31]. It is believed that the inflammation arises as a result of the release of cartilage breakdown products into the synovial fluid [32, 33]. Synovitis has been observed in both early and advanced OA [34-36]. There is increased expression of proinflammatory cytokines and activated B and T cell infiltration [31, 37, 38].

Cytokines are soluble or cell surface molecules that mediate cell-cell interactions. These molecules generally act locally at specific tissue sites through either a paracrine or autocrine mechanism. Cytokines can regulate both homeostatic chondrocyte remodeling and pathologic degradation and are classified as catabolic (IL-1β, TNFα, IL-17, IL-18), anti-catabolic (IL-4, IL-10, IL-13), anabolic (IGF-1, TGFβ, BMP-2), and modulatory (IL-6, IL-11) [31, 39-41].

The catabolic cytokines IL-1β and TNFα are the classical inflammatory mediators associated with cartilage matrix degradation in OA [42]. Both cytokines decrease proteoglycans synthesis and increase aggrecan release by the induction of MMPs and ADAMTS-4 and -5. In addition, IL-1β also induces the secretion of other

catabolic or anabolic modulators including CXCL8, IL-6, NO and PGE_2 [11, 30, 43]. IL-17 and IL-18 are also effective inducers of cartilage catabolism by increasing chondrocyte-generated IL-1β expression, stimulating the production of NO, MMPs and aggrecanses, and suppressing PG synthesis [47-50].

In a model of OA where the medial meniscus is partially removed, mice deficient in IL-1β or IL-1 converting enzyme demonstrated accelerated cartilage destruction compared to wild-type and not the cartilage protection that was theorized [44]. One explanation for this discrepancy could be that IL-1β treatment has been shown to enhance chondrocyte mediated release of the anabolic factor bone morphogenic protein-2 (BMP-2) whereas the anabolic cytokines TGFß and IGF-1 had no effect on the synthesis of BMP-2 [45]. Another explanation could be the OA model used was too severe to see protection since IL-1β knockout mice were protected from the destruction observed in the wild-type cartilage in a less severe model employing the disruption of the meniscus [46].

The simultaneous increase in IL-1β or TNFα-induced catabolic and anabolic activity increases matrix turnover both physiologically and pathologically. However, in the pathologic condition of OA, extended exposure to these proinflammatory cytokines favors catabolism leading to increased cartilage degradation.

The chemokine family of inflammatory mediators consists of approximately 50 different members and closely resemble cytokines [51]. Normal chondrocytes express some chemokines and chemokine receptors and this expression is increased in OA cartilage [52]. IL-1β, TNFα and IL-17 up-regulate the chondrocyte-derived secretion of GROα/CXCL1, IL-8/CXCL8, and MCP-1/CCL2 [53-55].

Concentrations of IL-8/CXCL8 and MCP-1/CCL2 are greater than those of IL-1β and TNFα in normal and OA cartilage explants [29, 51]. RANTES/CCL5 stimulated chondrocyte expression of MMPs, increased GAG release and NO generation, and inhibited PG synthesis in cartilage explants [56]. Additionally, IL-8/CXCL8 and GROα/CXCL1 induced human chondrocytes to differentiate and calcify and promoted an increase in NO release and MMP-13 activity [57].

Inducible nitric oxide synthase (iNOS) stimulates NO production in chondrocytes in response to IL-1β and TNFα [31]. NO has been shown to inhibit aggrecan synthesis, increase MMP activity, enhance chondrocyte susceptibility to oxidative damage, and modulate chondrocyte apoptosis [58].

1.2.3 Risk factors of OA

OA affects approximately 60% of men and 70% of women above the age of 65 [9] and is the most strongly correlated OA risk factor. The age-related cartilage damage is a result from the inability of the aging chondrocyte to maintain and repair the tissue. Chondrocyte mitotic and synthetic function declines with age leading to decreased and irregularly formed cartilage matrix [60]. Factors that influence the age-related reduction in chondrocyte function include senescence, decreased telomere length and oxidative damage [61].

Another important risk factor for OA is obesity [62, 63]. The joint degeneration seen in obese patients results from an increase in mechanical load on weight-bearing

joints [63]. A greater body mass index for both genders has been associated with increased radiographic evidence of OA in the knee but not the hip [64].

Genetic predisposition for OA can occur in some families. Genome-wide linkage analyses have revealed polymorphisms in genes that may determine susceptibility to OA at specific sites. Mutations in extracellular matrix proteins including collagens type II, IV, V and VI, cartilage oligomeric matrix protein, and aggrecan have been associated with early-onset OA [65]. In addition, variations in the anabolic factor IGF-1 and the catabolic factor IL-1β have been associated with OA-susceptibility [66].

A previously injured joint is highly predisposed to the development of OA. Repetitive, high impact sports are strongly associated with OA as articular cartilage is not resistant to such intense loading [67]. Under physiologic conditions of impact loading, the thin articular cartilage is protected by the lengthening of the muscles surrounding the joint and the underlying subchondral bone [68]. Excessive loads may damage the subchondral bone, resulting in more rigid bone with less effective shock absorption. The articular cartilage is then subject to extreme impact loading eventually leading to cartilage destruction [69]. However, it is still unclear if subchondral bone sclerosis precedes OA or is a result of the cartilage degeneration.

There are distinct gender differences in the predisposition for OA. Women over 50 years are twice as likely as men to develop OA, have more joints involved and are more likely to express clinical symptoms [70]. The predilection for OA in women after age 50 may result from postmenopausal estrogen deficiency and hormone replacement therapy has resulted in a decreased risk for knee and hip OA [71].

1.3 Animal Models of OA

Spontaneous OA occurs in various strains of mice, guinea pigs, and Syrian hamsters [72]. Though the pathogenesis of spontaneous animal OA is similar to the most common forms of human OA, it is a slow, progressive form of the disease. Therefore, surgically-induced instability models have been employed that mimic traumatic OA observed in humans. These models of OA progress more rapidly than spontaneous forms [73].

Medial meniscal tear in rats is performed by transection of the medial collateral ligament with a single full-thickness cut of the meniscus. Three to six weeks after surgery, significant cartilage degradation is demonstrated by increased surface fibrillation, loss of proteoglycans, osteophyte formation, and increased chondrocyte clustering [74].

Partial meniscectomy (PM) in rats and rabbits involves the transection of the lateral collateral ligament and excision of the medial meniscus. Cartilage in this model is marked by severe chondrocyte and proteoglycans loss, with significant surface fibrillation, osteophyte formation and subchondral bone sclerosis [73].

Transection of the anterior cruciate ligament (ACL-T) can be performed through a typical surgical incision or a blind "stab" incision. This model has been well characterized in dogs, but has also been performed on rats [73]. In the rat model, loss of superficial chondrocytes was observed by 2 weeks post-surgery and small surface fibrillations were observed by 4 weeks. By 10 weeks, the cartilage exhibited severe fibrillations, loss of proteoglycans and chondrocyte cloning [75].

To induce OA more rapidly, PM and ACL-T have been used in combination. Surface fibrillations and loss of proteoglycans was observed by 2 weeks post-surgery. When the rats were then forced to move after the surgery, the onset and progression of OA was more severe, i.e. by 12 weeks there was significant subchondral bone sclerosis in the forced mobilization group compared to 20 weeks in the non-mobilized group [76].

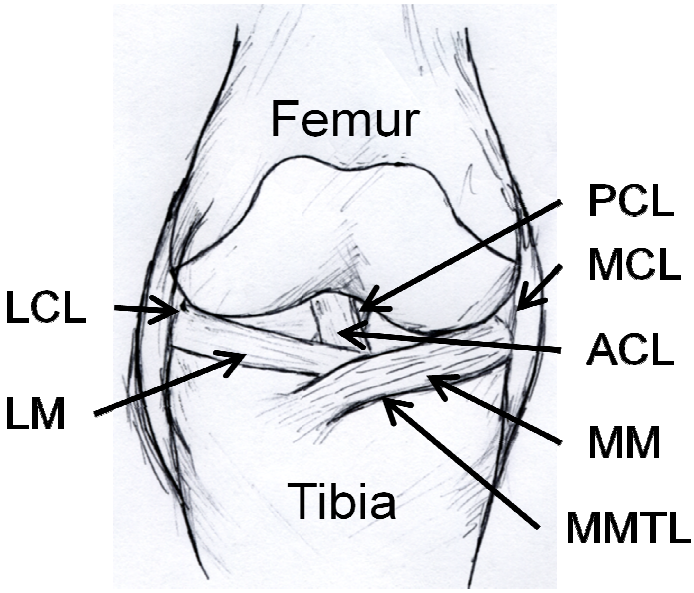


FIGURE 1.3
Schematic of the anatomy of the knee joint. LCL: lateral collateral ligament; LM: lateral meniscus; PCL: posterior cruciate ligament; MCL: medial collateral ligament; ACL: anterior cruciate ligament; MM: medial meniscus; MMTL: medial meniscotibial ligament.

These surgically-induced instability models were originally performed on larger rodents, sheep and dogs. Now with specific microsurgical techniques, instability can be induced in the mouse knee joint (Figure 1.3). By applying different combinations of ligament transection and meniscectomy, Kamekura, *et al*. have demonstrated varying severities of mouse OA [77]. The severe model was performed by transection of the anterior/posterior cruciate ligaments and the medial/lateral collateral ligaments followed by the removal of the medial/lateral menisci. The moderate model was the PM/ACL-T

described above and the mild model was the ACL-T. After 2 weeks, joint destruction was observed in the severe model, which was not present in the moderate model until 4 weeks and the mild model until 8 weeks. Glasson, *et al.* have developed a milder model of OA where the medial meniscotibial ligament is transected, disrupting the medial meniscus (DMM) leading to loss of proteoglycans and increased surface fibrillations at 8 weeks post-surgery [78].

1.4 The Receptor for Advance Glycation Endproducts

RAGE is a member of the immunoglobulin superfamily of cell surface receptors. The full length receptor is composed of three immunoglobulin-like regions: one V-type ligand-binding domain, two C-type-domains, a short transmembrane domain, and a 43-amino acid cytoplasmic tail which is critical for intracellular signaling [79].

Other RAGE isoforms that undergo alternative RNA splicing have been described [80]. These include an N-truncated form which lacks the V-type domain and cannot bind certain ligands and a C-truncated form that lacks the transmembrane domain (endogenous secretory [esRAGE]). The total extracellular soluble RAGE (sRAGE) found in plasma is comprised of esRAGE and a RAGE molecule that has been proteolytically cleaved from the surface by matrix metalloproteinases [81]. By competing with cell-surface RAGE for ligand binding, sRAGE may function as a decoy receptor by sequestering circulating ligands [80].

Since it recognizes widely different classes of ligands, RAGE is considered to be a pattern recognition receptor. RAGE was originally found as the receptor for Advanced Glycation End-products (AGE's) [82], but subsequently described ligands include amyloid-β, HMGB1 (amphoterin), advanced oxidation protein products, as well as S100/calgranulins [83-87]. RAGE also interacts with surface molecules on bacteria, prions, and leukocytes [88-90].

Cell-surface RAGE is expressed at high levels during development then decreases to lower levels in adult tissue [80]. RAGE is expressed in various cell types, including macrophages, endothelial cells, smooth muscle cells, myocytes, neuronal cells, and chondrocytes [91, 92]. Though expression in adult tissue is normally low, RAGE can

become upregulated in specific areas where its ligands accumulate, such as in the inflammatory environment of diabetes, atherosclerosis, Alzheimer's disease, rheumatoid arthritis and chronic renal disease [93, 94]. The inflammatory response is then amplified as RAGE signaling activates the proinflammatory transcription factor NF-κB. Furthermore, NF-κB activation also increases RAGE expression [95].

RAGE expression has been shown to increase in aging tissue, and recent evidence has associated RAGE expression with both age and the presence of OA pathology in humans [91]. The involvement of RAGE in the pathology of chronic disease has been demonstrated in murine models using sRAGE, RAGE neutralizing antibodies, or a dominant-negative form of the receptor as well as in $RAGE^{-/-}$ mice [96-99].

1.5 S100/Calgranulins

There have been 25 members of the S100/calgranulin family of proteins identified to date [100]. Most S100/calgranulins have both intracellular and extracellular functions including cell growth and differentiation, cell cycle regulation, transcription, signal transduction, and chemotaxis [101]. The monomers of most S100/calgranulins are between 9 and 14 kDa and are comprised of two EF-hand calcium-binding subunits, one canonical and one unique to calgranulins.

S100/calgranulins normally exist inside the cell as noncovalent homodimers positioned in an anti-parallel position [102]. Some members of the S100 family, such as S100A8 and S100A9, form heterodimers [103]. Dimerization of S100 proteins seems important for their intracellular activities: Ca^{2+} binding induces a conformation change allowing each S100 monomer to ligate a target protein. Additionally, the S100 dimer can bind target proteins on opposite sides, thereby functionally crosslinking two proteins [102].

No S100 protein appears to be expressed in all tissues, suggesting that the relatively large number of members is not simply due to redundancy but due to specific tissue expression and functional localization [102]. One unusual characteristic of certain S100 proteins, such as S100B, S100A4, S100A8, S100A9 and S100A12 is their accumulation in the extracellular space at sites of inflammation where they act in a cytokine-like manner. The S100A8/A9 heterodimer is a chemotactic molecule in inflammation, S100B initiates neurite outgrowth, S100A4 induces angiogenesis in tumor growth and influences MMP-13 activity, and S100A12 is involved in the inflammatory

response [84, 104-106]. The multiligand receptor for advanced glycation endproducts (RAGE) has been identified as mediating these extracellular activities [82].

S100A11 was used as a prototypical ligand for RAGE in this dissertation since there was an increased expression in a microarray analysis of osteoblasts from the hypermineralizing NPP1$^{-/-}$ mouse (K. Johnson, unpublished observation). This calgranulin was originally cloned from the chicken gizzard [107] and is primarily expressed in mesenchymal-derived cells of the skin, kidney, gastrointestinal tract, reproductive tract, cartilage and smooth muscle [108].

Intracellular S100A11 regulates the association of annexin I with the extracellular membrane [109], and inhibits actin-activated myosin ATPase activity [110], affecting the organization of F-actin. S100A11 is upregulated in many different types of human cancer. Its effects have not been completely elucidated [111], but it has been suggested that S100A11 is a tumor suppressor affecting contact inhibition of cell growth [102]. In addition, studies have demonstrated that intracellular S100A11 inhibits growth of keratinocytes, whereas extracellular S100A11 stimulates growth [112].

1.6 Transglutaminase 2

All transglutaminases (TG) share the common enzymatic activity of transamidation: the cross-linking of glutamine and lysine residues to form N epsilon (gamma-glutamyl) lysyl isopeptide bonds. Each TG enzyme is localized and acts on specific substrates. To date, nine members of the TG family have been identified, but only TG2 and FXIIIA have been detected in cartilage [113, 114].

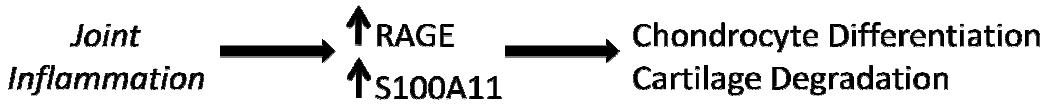
Although TG2 lacks a leader sequence, it is still secreted and can affect the stabilization of the extracellular matrix (ECM). Additionally, TG2 has the potential to interact with other secreted proteins in the extracellular matrix [115].

The release of TG2 and accumulation in the extracellular matrix is dramatically increased in the cartilage of OA patients and synovial fluid of osteoarthritic guinea pigs [116]. After cartilage injury, increased TG2 transamidation activity has been proposed to help stabilize the ECM and prevent further proteolytic or mechanical damage [117, 118]. In addition to its transamidation activity, TG2 has the ability to bind and hydrolyze both GTP and ATP. It has been demonstrated recently that this function of TG2 is critical for the induction of chondrocyte hypertrophic differentiation [119].

Several cartilage-derived substrates for TG2 transamidation have been described, including fibronectin, collagen, osteocalcin, and osteopontin [120]. TG2 transamidation sites in S100A11 have also been described [121]. Furthermore, a mechanism of protein oligomerization has been described for S100A10 and S100A11 where TG2 promotes the formation of covalently linked homomultimers [122]. Multimeric forms of S100A12, S100A4, and S100B appear to be associated with their extracellular activity by initiating an aggregation of RAGE [123-125].

1.7 Hypothesis

Inflammation-induced expression of RAGE and S100A11 associated with OA stimulates chondrocyte hypertrophic differentiation and dysregulated cartilage matrix catabolism.



Chapter 2 Hypothesis:

S100A11 signals through RAGE and p38 MAPK to induce chondrocyte hypertrophic differentiation. Using sRAGE and RAGE blocking antibodies, S100A11-induced differentiation on human chondrocytes was evaluated.

Chapter 3 Hypothesis:

The TG2 transamidation of S100A11 induces a conformational change critical to stimulate chondrocyte differentiation and cartilage catabolism. S100A11 is a substrate for TG2 transamidation, and both TG2 and S100A11 play a role in the progression of OA. The interaction between TG2 and S100A11 using wild-type, RAGE null and TG2 null mouse chondrocytes and cartilage explants was examined.

Chapter 4 Hypothesis:

RAGE directly mediates the pathogenesis of murine instability-associated knee OA in vivo. After inducing OA by the "stab" model of ACL-T, the role of RAGE was assessed in overall joint score, osteophyte formation, cartilage appearance and PG loss.

CHAPTER 2

Inflammation-Induced Chondrocyte Hypertrophy Is Driven by Receptor for Advanced Glycation End Products

2.1 ABSTRACT

The multiligand receptor for advanced glycation end products (RAGE) mediates certain chronic vascular and neurologic degenerative diseases accompanied by low-grade inflammation. RAGE ligands include S100/calgranulins, a class of low-molecular-mass, calcium-binding polypeptides, several of which are chondrocyte expressed.

Here, we tested the hypothesis that S100A11 and RAGE signaling modulate osteoarthritis (OA) pathogenesis by regulating a shift in chondrocyte differentiation to hypertrophy. We analyzed human cartilages and cultured human articular chondrocytes, and used recombinant human S100A11, soluble RAGE, and previously characterized RAGE-specific blocking Abs. Normal human knee cartilages demonstrated constitutive RAGE and S100A11 expression, and RAGE and S100A11 expression were up-regulated in OA cartilages studied by immunohistochemistry. CXCL8 and TNFα induced S100A11 expression and release in cultured chondrocytes. Moreover, S100A11 induced cell size increase and expression of type X collagen consistent with chondrocyte hypertrophy in vitro. CXCL8-induced, IL-8-induced, and TNFα induced but not retinoic acid induced chondrocyte hypertrophy was suppressed by treatment with soluble RAGE or RAGE-specific blocking Abs. Last, via transfection of dominant-negative RAGE and dominant-negative MAPK kinase 3, we demonstrated that S100A11 induced chondrocyte type X collagen expression was dependent on RAGE mediated p38 MAPK pathway activation.

We conclude that up-regulated chondrocyte expression of the RAGE ligand S100A11 in OA cartilage, and RAGE signaling through the p38 MAPK pathway,

promote inflammation-associated chondrocyte hypertrophy. RAGE signaling thereby has the potential to contribute to the progression of OA.

2.2 INTRODUCTION

Low-grade chronic inflammation, mediated partly by the effects on chondrocytes of cartilage-expressed and synovium-expressed cytokines including IL-1β, TNFα, (IL-8)/CXCL8, and (growth-related oncogene α)/CXCL1 appear to contribute to the progression of osteoarthritis (OA) [2, 31, 52, 57, 126-129]. Recently, we linked the induction of altered chondrocyte differentiation to chemokine-induced inflammation, by demonstrating that CXCL8 and CXCL1, both of which are up-regulated in OA cartilage [52], stimulated chondrocyte hypertrophic differentiation in vitro [57]. Chondrocyte hypertrophy can contribute to the progression of OA via effects including dysregulation of matrix repair through reduced expression of collagen II and aggrecan, increased expression of type collagen X, up-regulation of matrix metalloproteinase 13 (MMP-13), and promotion of pathologic calcification [23, 57, 130]. OA cartilages typically develop foci of maturation of cells to hypertrophic differentiation [23].

A growing body of evidence has implicated the receptor for advanced glycation end products (RAGE) in certain chronic arterial, renal, and neurologic degenerative conditions associated with low-grade tissue inflammation [84, 131-133]. RAGE is a broadly expressed 45-kDa transmembrane protein bearing a 43-aa cytosolic tail, and three extracellular domains (i.e., the ligand-binding V (V') domain, and two C domains) that confer RAGE membership in the Ig superfamily [84, 131-133]. RAGE is a cognate receptor for four distinct classes of ligands [84, 131-133], including S100/calgranulins, a family of >20 low-molecular-mass (~10–14 kDa), acidic proteins that form homodimers, heterodimers, and larger multimers, and bind calcium via two internal EF-hand motifs in each monomer [84, 100, 134]. Certain individual S100/calgranulins (S100A1, S100A2,

S100A4, S100B) have been reported to be expressed by chondrocytes [91, 135, 136] and intense S100 immunoreactivity is a marker of chondrogenic differentiation [137]. In addition, concentrations of certain calgranulins in diseased joint fluids reach nanomolar concentrations [138, 139].

Recently, RAGE expression was demonstrated in human articular cartilage and observed to be more robust in aging and OA cartilages [91]. Furthermore, IL-1β and fibronectin fragments induced chondrocyte RAGE expression and micromolar S100B induced MMP-13 in chondrocytes mediated by RAGE in vitro [91]. In this study, we examined the potential role in articular chondrocyte hypertrophy of RAGE, with particular focus on RAGE interaction with S100A11 (S100C, calgizzarin) [100, 134]. Our results implicate up-regulated S100A11 expression, and RAGE-dependent and p38 MAPK-dependent signaling, in inflammatory regulation of chondrocyte hypertrophic differentiation in OA cartilage.

2.3 RESULTS

RAGE and S100A11 expression in OA cartilage in situ.

S100/calgranulin sequences contain several highly conserved elements [100]. Hence, for these studies, we generated murine polyclonal Abs to the C-terminal 15-aa peptide of the calgranulins RAGE ligand S100A11, a region C-terminal to the canonical EFhand domain (Fig. 1A) and that was determined to have a unique sequence by BLAST analysis, including comparison aligned C termini of other S100/calgranulins (Table 2.1). "Dot-blot" immunoblotting confirmed specificity of the Ab for S100A11 (Fig. 2.1B).

Immunohistochemical analyses revealed low-level constitutive expression of RAGE and S100A11 in normal human knee articular cartilages (Fig. 2.2). We confirmed [91] that chondrocyte RAGE expression appeared up-regulated in OA knee cartilages, particularly so in the deep zone but also as detected in chondrocytes in the superficial zone (Figure 2.2). S100A11 expression was markedly increased in all zones of OA cartilages (Figure 2.2). S100A11 became particularly abundant in the pericellular matrix of OA chondrocytes (Figure 2. 2), suggesting that chondrocytes actively secreted S100A11 in the course of OA.

Induction of S100A11 in cultured human articular chondrocytes.

Cultured normal human knee articular chondrocytes were stimulated for 24 h with the known inducers of chondrocyte hypertrophy CXCL8 and ATRA [57, 130, 140]. We also stimulated chondrocytes with TNFα, a modulator of OA pathogenesis [2, 31]. As assessed by flow cytometry, RAGE was expressed by 59 ± 9% of normal articular chondrocytes (n = 6), with no detectable, significant effects on RAGE expression of

CXCL8, ATRA, or TNFα (Figure 2.3A). Concurrent analysis of the normal articular chondrocytes by SDS-PAGE/Western blotting revealed that S100A11, which remained below limits of detection in both unstimulated and stimulated cell lysates (not shown), was both robustly induced and released into the conditioned medium by chondrocytes in response to CXCL8, ATRA, and TNFα (Figure 2.3B). S100A11, like certain other calgranulins, is known to form covalently bonded dimers and multimers and this process optimizes binding to RAGE [100, 121, 123]. Whereas S100A11 isolated from 293 cells was detectable only as a monomer of ~11 kDa, S100A11 in conditioned medium of chondrocytes treated with exogenous S100A11, or with CXCL8, ATRA, and TNFα, appeared partially multimeric, with particularly large multimers detected in CXCL8-treated cells (Figure 2.3B).

S100A11-induced chondrocyte hypertrophy mediated by RAGE.

Because the release of S100A11 was inducible in normal chondrocytes, we next tested for the potential of S100A11 to regulate chondrocyte differentiation. We observed that 10 nM S100A11 (100 ng/ml) induced type X collagen expression in human articular chondrocytes after 5 days in culture (Figure 2.4A). Dose-response studies indicated detectable induction of type X collagen by 100 ng/ml S100A11 but not 0.1–10 ng/ml S100A11 (data not shown). Chondrocytes coincubated with sRAGE, which lacks the transmembrane and cytosolic tail domains of RAGE [141], demonstrated attenuated type X collagen expression in response to 100 ng/ml S100A11 (Figure 2.4A). Because sRAGE has the potential to affect the binding of RAGE ligands to receptors other than RAGE, we

additionally studied the effects of chondrocyte treatment with 20 μg/ml previously described rabbit polyclonal blocking Abs specific for RAGE [141]. We used conditions under which 20 μg/ml nonimmune rabbit IgG was confirmed to not suppress the induction of type X collagen (data not shown). Under these conditions, the RAGE-specific blocking Abs were observed to suppress S100A11-induced chondrocyte type X collagen expression (Figure 2.4A). Next, we validated induction of chondrocyte hypertrophy by examining the chondrocytes for S100A11-induced size increase. Within 24 h, >20% of chondrocytes increased detectably beyond the resting size range in response to S100A11, as assessed by increase in forward scatter via flow cytometry analysis (Figure 2.4B). These changes in chondrocyte size in response to S100A11 were also inhibited by both sRAGE and RAGE-specific blocking Abs (Figure 2.4B).

RAGE mediation of inflammatory cytokine-induced but not ATRA-induced chondrocyte hypertrophy.

We confirmed [57, 130, 140] the induction of cultured articular chondrocyte hypertrophy by CXCL8 and ATRA, and we concurrently observed that type X collagen expression induction by CXCL8 but not ATRA was attenuated by coincubation with sRAGE or with RAGE-specific blocking Abs (Figure 2.5, A and C, respectively). Concomitantly, we discovered that TNFα induced chondrocyte type X collagen expression and that TNFα did so in a manner attenuated by sRAGE or RAGE-specific blocking Abs (Figure 2.5B).

S100A11 signaling through RAGE to induce p38 MAPK kinase pathway activation and type X collagen.

We previously demonstrated that p38 MAPK pathway activation was essential for transduction of chondrocyte hypertrophy in response to CXCL8 and CXCL1 [57]. Hence, we concluded the study by assessing the specific role of RAGE signaling via the p38 MAPK pathway in S100A11-induced type X collagen expression. We observed that S100A11 stimulated rapid p38 phosphorylation in human chondrocytes (Figure 2.6A). S100A11-induced p38 activation was inhibited by transfection of chondrocytes with a DN construct of MKK3 (DN-MKK3), a specific inhibitor of the highly selective p38 pathway activation inducer MKK3 [142] (Figure 2.6B). DN-MKK3 also inhibited type X collagen expression induction by S100A11 (Figure 2.6B). Because RAGE is not the sole plasma membrane receptor for S100/calgranulins [100, 143, 144], we assessed the effects of specific inhibition of RAGE signal transduction using DN RAGE (DN-RAGE). Transient transfection of DN-RAGE attenuated both S100A11-induced p38 phosphorylation and type X collagen expression in human articular chondrocytes (Figure 2.6C).

2.4 DISCUSSION

In this study, we demonstrated marked up-regulation of expression of the RAGE ligand S100A11 within human OA cartilage. S100/calgranulins exert a variety of physiologic effects on cell function, mediated partly by calcium binding and calmodulin-like activities, complex formation with selected annexins and other proteins, and intracellular translocation [100, 134, 136, 138]. For example, S100A11 has been implicated in regulatory effects of TGFα on cell growth, via induction by TGFα of protein kinase C-mediated S100A11 phosphorylation and nuclear S100A11 translocation [145]. But, increasingly, certain S100 proteins have been observed to be secreted and to exert extracellular effects, illustrated by proinflammatory effects on leukocyte recruitment by S100A8, and S100A9 [132, 143], and by proinflammatory RAGE-dependent effects of S100A12 (extracellular newly identified RAGE binding protein) on endothelial cell and macrophage cytokine release and adhesion [84]. In this study, we demonstrated that CXCL8, TNFα, and ATRA induced chondrocytes to release S100A11. Moreover, we observed that exogenous nanomolar rS100A11 was sufficient to promote chondrocyte hypertrophy, as evidenced by both type X collagen expression and cell size increase.

In this study, we observed that RAGE was constitutively expressed by normal articular chondrocytes in situ, with the qualitative appearance of up-regulated chondrocyte expression of RAGE by chondrocytes in OA cartilage, confirming recently published observations [91]. S100/calgranulins bind not only RAGE but also CD36 [144]. S100/calgranulin-mediated cellular effects via binding to cell surface proteoglycans also have been demonstrated [144]. However, using an established

approach based on parallel results of treatment with sRAGE or with function-blocking Abs to block RAGE ligand-induced signaling [141], we demonstrated that both S100A11-induced chondrocyte size increase and expression of type X collagen were RAGE mediated.

The prior observation that the chemokines CXCL8 and CXCL1 induce cultured articular chondrocyte hypertrophy [57] suggested a novel mechanistic linkage between low-grade joint inflammation and dysregulated chondrocyte differentiation within OA cartilage [2, 31, 52, 57, 126-129]. An unexpected finding in this study was that CXCL8- and TNFα-induced type X collagen expression also were inhibited via sRAGE and RAGE-specific blocking Abs. This result suggested that S100A11, and likely other RAGE ligands induced by CXCL8 and TNFα, were critical downstream mediators of chondrocyte hypertrophy in response to IL-8 and TNFα, as schematized in Figure 2.7. Importantly, under conditions in which RAGE mediated CXCL8- and TNFα-induced chondrocyte hypertrophy and in which ATRA induced S100A11, we observed no suppression of ATRA-induced expression of type X collagen expression. We speculate that chondrocyte hypertrophy in response to the growth and differentiation mediator ATRA is driven by more redundant mechanisms [130] than is the hypertrophy response to the inflammatory cytokines CXCL8 and TNFα. Using transfection of exquisitely selective DN inhibitors of RAGE and of p38 MAPK pathway activation, we demonstrated that activation of the p38 MAPK pathway via RAGE-dependent signaling was essential for transduction of chondrocyte hypertrophy in response to S100A11. We previously observed that CXCL8-induced p38 MAPK pathway activation and

chondrocyte hypertrophy [57] are critically mediated by stimulation of Pit-1 expression and increased sodium-dependent phosphate cotransporter-mediated Pi uptake [140]. Hence, it will be of interest to test potential roles of Pit-1 expression, and Pi uptake in S100A11- induced and RAGE-mediated chondrocyte hypertrophy. Significantly, RAGE ligands of the advanced glycation end products (AGE) class accumulate in tissues in association with both normal aging and diabetes mellitus [131, 132] and also form by posttranslational modification of collagen and other extracellular matrix proteins in association with cartilage aging and OA [146]. AGE recognition by RAGE is a central mediator of diabetic microvascular, renal, and neuropathologic complications [131, 132]. Moreover, AGE-induced effects on chondrocytes have been linked to decreased proteoglycans and collagen synthesis in vitro [146]. Certain biological responses to the chromatin-binding protein amphoterin/high-mobility group box-1 (HMGB-1) are mediated directly by RAGE [86]. HMGB-1 is expressed by articular chondrocytes [86], and secreted HMGB-1 can exert proinflammatory cytokine-like effects and has been implicated as an inflammatory mediator in rheumatoid arthritis [147]. We have observed induction of type X collagen by AGEs, HMBG-1, and S100B in cultured chondrocytes (D. L. Cecil and R. Terkeltaub, unpublished observations), and it was recently reported that HMGB-1 and S100B induced MMP-13 expression in primary chondrocytes mediated by RAGE-dependent signaling [91]. Therefore, in the model of inflammation-stimulated chondrocyte hypertrophy schematized in Figure 2.7, we propose that RAGE ligands including S100A11 synergize in promoting altered chondrocyte differentiation and function when externalized by activated chondrocytes.

Limitations of this study included the use of first-passage as opposed to primary chondrocytes. We did not investigate potential effects on chondrocyte differentiation of intracellular S100A11, an inhibitor of cell growth, and regulator of cell senescence and apoptosis [148, 149]. Because p38 pathway activation, but not activation of ERK1/2 or JNK, is central to CXCL8-induced chondrocyte hypertrophy [57], we limited mechanistic studies here to the involvement of p38 pathway activation in chondrocyte hypertrophic differentiation mediated by nanomolar S100A11. The current study did not assess potential effects of S100A11-induced chondrocyte activation of ERK1/ERK2 pathway and NF-κB, which were recently reported to modulate chondrocyte activation by S100B added at micromolar concentration [91]. We did not monitor for potential constitutive or stimulated chondrocyte release of endogenous sRAGE [150] or for potential function-regulating effects of chondrocyte-derived glycosaminoglycans binding by the RAGE extracellular domain [151] or possible effects of chondrocyte MMPs on RAGE extracellular domain attachment to the plasma membrane [150].

Additionally, S100A11, like several other calgranulins, is a substrate for transglutaminase-dependent covalent modification, with the reactive regions in S100A11 defined to be at function-specifying N- and C-terminal domains [121, 122]. In this context, we observed S100A11 multimerization in the conditioned medium of cultured chondrocytes in this study, a process for S100A11 that is catalyzed by transglutaminase 2 (TG2) [121, 122] and that promotes the capacity of certain S100/calgranulins to activate signaling via RAGE [100, 123]. TG2 is externalized by activated chondrocytes and plays a major role in mediating both CXCL8 induced and ATRA-induced chondrocyte

hypertrophy [57, 130]. Hence, it will be of interest to test the direct role of TG2 in S100A11-induced chondrocyte hypertrophy.

We conclude that there is accumulation in OA articular cartilages of the RAGE ligand S100A11. RAGE-dependent signaling in response to not only S100A11 but also CXCL8 and TNFα, promotes chondrocyte hypertrophic differentiation and thereby may factor into contributory effects of low-grade inflammation to OA progression.

The text of chapter 2 is a reprint of the material as it appears in the Journal of Immunology, Cecil DL, Johnson K, Rediske J, Lotz M, Schmidt AM, Terkeltaub R. "Inflammation-induced chondrocyte hypertrophy is driven by receptor for advanced glycation end products", 175(12):8296-302, 2005. I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis of this chapter.

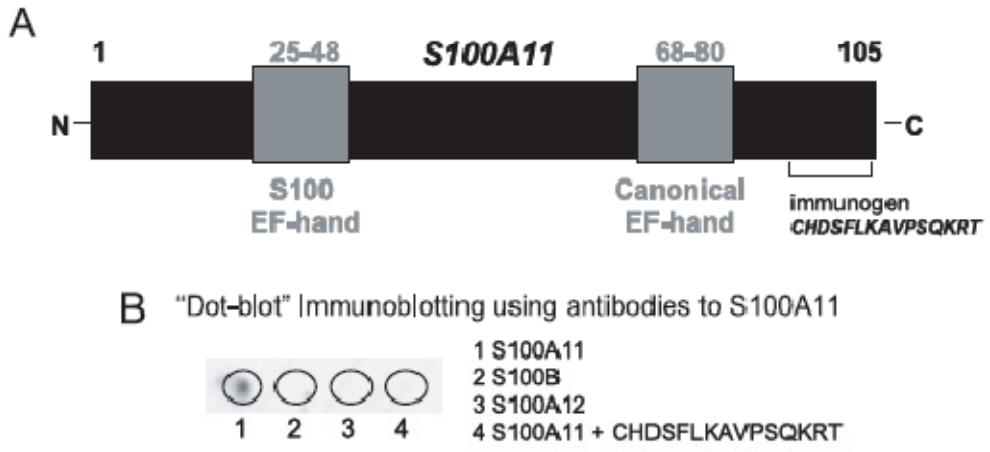


FIGURE 2.1
Specificity of the murine anti-peptide polyclonal S100A11. (A) Sequence specificity of S100A11 C terminal peptide sequence. The schematic of structural domains of S100A11 highlights a 15-aa peptide C-terminal to the second canonical S100/calgranulin EF hand used as the immunogen to make murine polyclonal Abs (please refer to Table I). (B) S100A11 Ab specificity verified by "dot blot" immunoblotting. Human S100A11, S100B, and S100A12 (250 ng each) were directly applied, as indicated, to nitrocellulose for immunoblotting, as described in Materials and Methods, and using the anti-peptide S100A11 Ab. Lane 4 contained 250 ng of S100A11 but was probed with the anti peptide S100A11 Abs after preadsorption with 5-fold molar excess to Abs of the unique immunogen S100A11 C-terminal peptide described above.

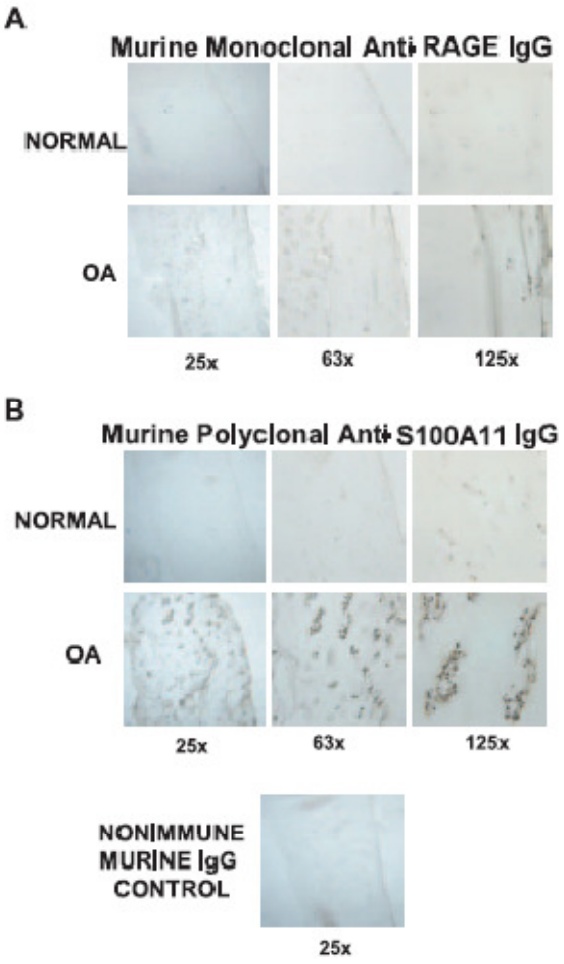


FIGURE 2.2
Comparison of expression of RAGE and S100A11 in normal and OA human knee articular cartilages. Frozen sections of normal and OA human knee articular cartilages were stained with murine monoclonal anti-RAGE Ab and with murine anti-peptide polyclonal Abs specific for S100A11, as described in Materials and Methods. Magnifications of x25, x63, and x116 are shown. The normal mouse serum control is shown at x25 magnification. These data are representative of three normal donors and three OA donors and demonstrate up-regulation of RAGE and, even more so, S100A11 in OA cartilages, as discussed in the text.

Table 2.1
Comparison of the aligned C-terminal amino acid sequences of S100A11 and 14 other S100/calgranulins. Alignment of C terminal amino acid sequences of the indicated S100/calgranulins by BLAST analysis is shown. The C-terminal 15-aa peptide sequence of S100A11 (in bold italics) was verified to be unique not only to calgranulins but also other proteins by BLAST analysis, and this peptide was the immunogen in generating murine polyclonal S100A11-specific Abs as described in Materials and Methods.

Protein	Alignment
S100B	[77] FVAMVTTACHEFFEHE[92]
S100A1	[76] LVAALTVACNNFFWENS[94]
S100A2	[78] FLALITVMCNDFFQGCPDRP[97]
S100A3	[78]SLACLCLYCHEYFKDCPSEPPCSQ[101]
S100A4	[78]FLSCIAMMCNEFFEGFPDKQPRKK[101]
S100A5	[93] FLTMLCMAYNDFFLEDNK[110]
S100A6	[76] FLGALALIYNEALKG[90]
S100A7	[78] LLGDIATDYHKQSHGAAPCSGGSQ[101]
S100A8	[74] LVIKMGVAAHKKSHEESHKE[93]
S100A9	[82] LMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP[114]
S100A10	[75] LIAGLTIACNDYFVVHMKQKGKK[97]
S100A11	[83] LIGGLAMA***CHDSFLKAVPSQKRT***[105]
S100A12	[77] LVAIALKAAHYHTHKE[92]
S100A13	[79] LIGELAKEIRKKKDLKIRKK[96]
S100A14	[87] LIGEAAKSVKLERPVRGH[104]

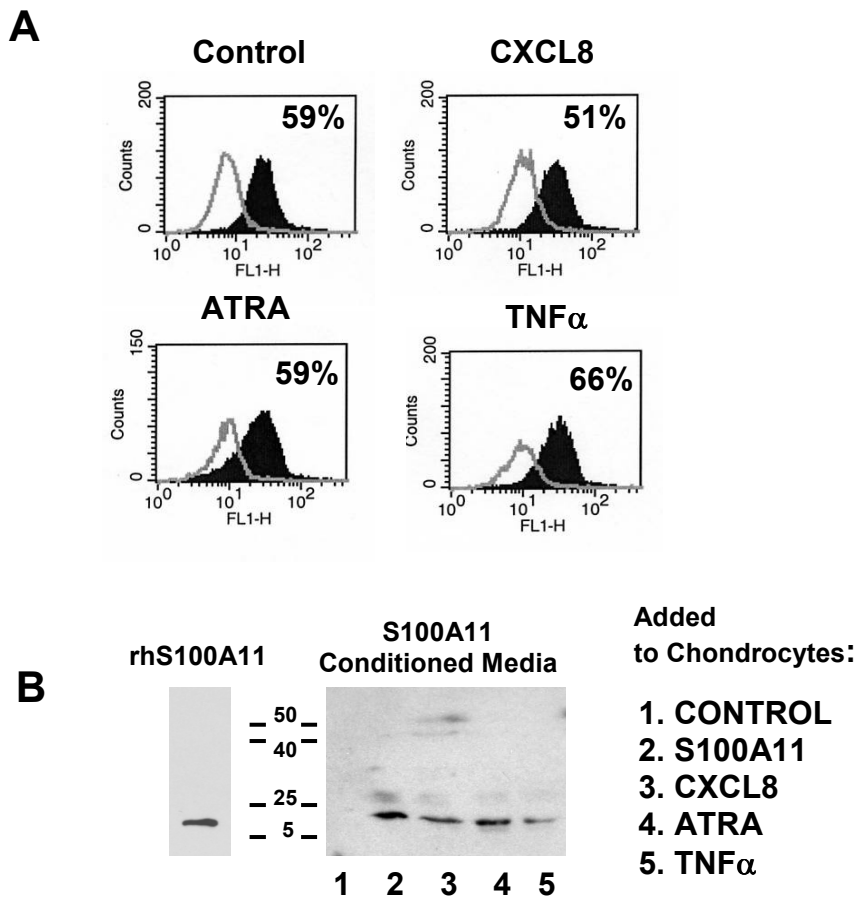


FIGURE 2.3

RAGE expression and inducible S100A11 secretion in cultured human articular chondrocytes. (A) RAGE expression. First-passage human knee articular chondrocytes (5 x 10^5 cells/six-well dish) from six normal donors (age range, 29–62), were prepared as described in Materials and Methods, were studied for RAGE expression by flow cytometric analysis. The panels, taken from studies of one normal donor representative of all the donors, depict binding of isotype control IgG (open histogram) and additionally of anti-RAGE IgG without stimulation or in response to CXCL8, TNFα, and ATRA for 24 h (solid histograms). In normal chondrocytes, constitutive RAGE expression was detected in 59 ± 9% of cells, and there were no significant changes in the proportions of RAGE-expressing chondrocytes in response to CXCL8, TNF-α, or ATRA, as seen in the representative results from the single donor demonstrated here. (B) S100A11 expression and multimerization in chondrocytes. SDS-PAGE/ Western blotting analysis was performed on 1 μg of human recombinant S100A11 isolated from transfected 293 cells as described in Materials and Methods (left side of figure), and on 30-μg aliquots of protein precipitated from conditioned medium of first-passage normal human knee chondrocytes (right side of figure) (donor ages, 29–62). The chondrocytes were prepared under the same conditions as for (A) above, and stimulated for 24 h by addition of S100A11, CXCL8, TNFα, or ATRA, at which time conditioned media were studied. Results are representative of those from six different normal human donors, as in A

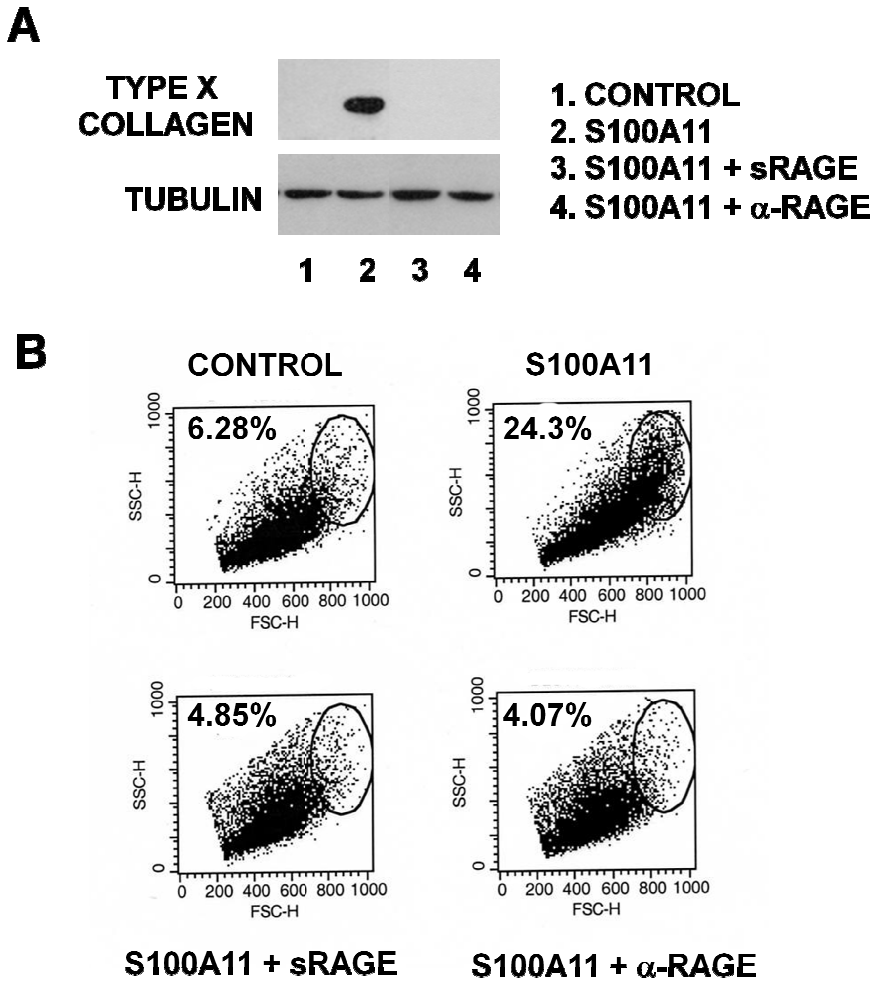


FIGURE 2.4
Effects of soluble S100A11 on type X collagen expression and chondrocyte size mediated by RAGE. First-passage normal human chondrocytes (donor ages, 22–62) (5 x 10^5 cells/six-well dish) were stimulated with S100A11 in the presence or absence of 1 μg/ml sRAGE or 20μg/ml RAGE-specific blocking Abs, where indicated. (A) SDS-PAGE/Western blotting analysis for type X collagen expression was performed using cell lysates at 5 days in culture. (B) Flow cytometry measurements of forward and side scatter were performed on the chondrocytes after 24 h of stimulation. These data are representative of results from eight normal human donors.

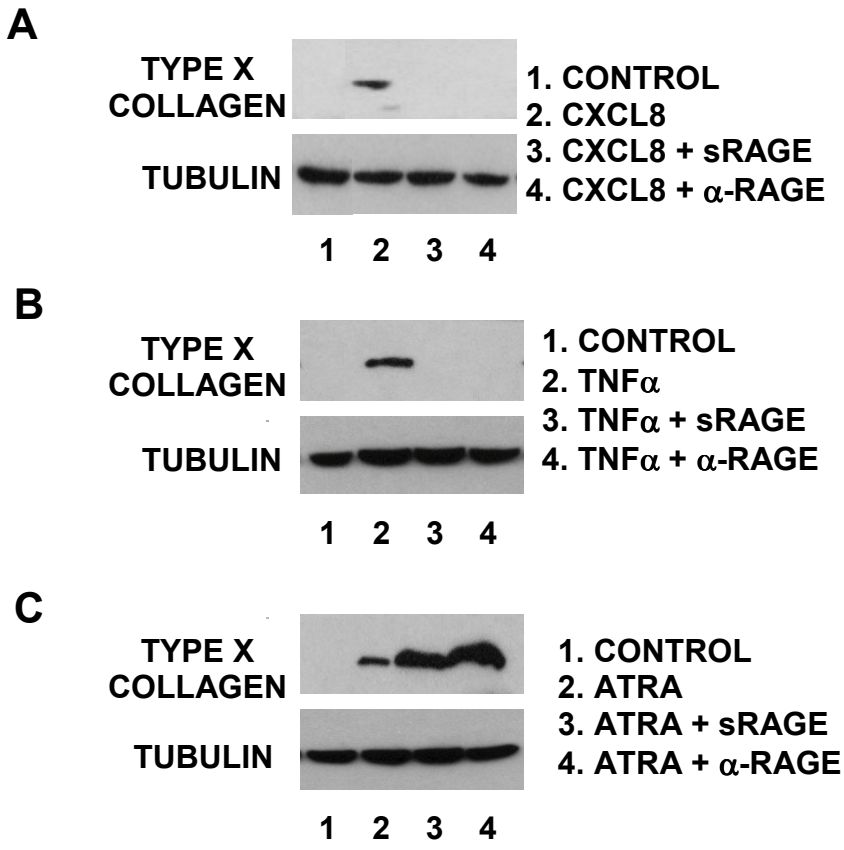


FIGURE 2.5
sRAGE and anti-RAGE inhibit CXCL8- and TNFα- but not ATRA-induced type X collagen expression. Primary normal human knee articular chondrocytes (donor ages, 29–62) (5 x 10^5 cells/six-well dish) were stimulated with CXCL8 (A) ATRA (B) or TNFα (C) in the presence or absence of 1 μg/ml sRAGE or 20 μg/ml anti-RAGE. SDSPAGE and Western blotting analysis for type X collagen were performed on cell lysates at 5 days in culture. These data are representative of results from six normal human donors.

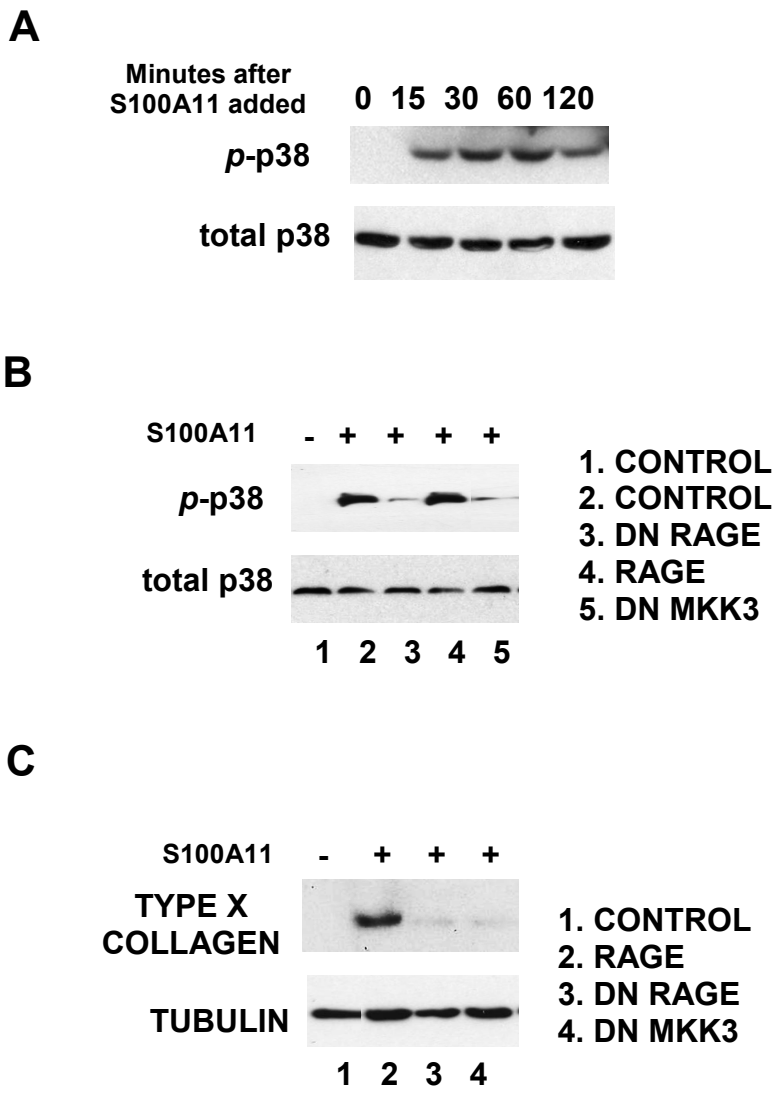


FIGURE 2.6

S100A11 signals through RAGE to induce p38 activity. Primary normal human chondrocytes (donor ages, 60–72) (1 x 10^6 cells/10-cm dish) were stimulated with S100A11 for the indicated time. SDS/PAGE and Western blotting analysis for phosphorylated p38 was performed on cell lysate. (A) Primary human chondrocytes (5 x 10^5 cells/six-well dish) were transfected with the indicated construct as described in Materials and Methods. The cells were stimulated with S100A11 for 15 min and SDS-PAGE/Western blotting was performed for phosphorylated p38 (B) and type X collagen (C). These data are representative of results from three to five different human donors.

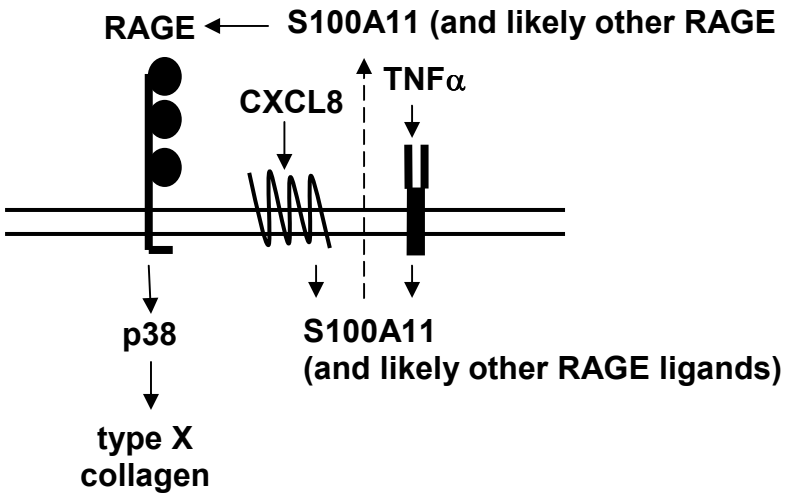


FIGURE 2.7
Schematic of RAGE signaling in the induction of chondrocyte hypertrophy. This schematic depicts the induction by CXCL8 and TNFα of secretion of S100A11 (and likely other chondrocyte-expressed RAGE ligands not limited to calgranulins). The paradigm further depicts subsequent RAGE signaling critically transduced by p38 MAPK pathway activation as a central event in chondrocyte hypertrophy associated with low-grade inflammation in the OA joint articular cartilage.

CHAPTER 3

Transamidation by Transglutaminase 2 Transforms S100A11 Calgranulin into a Pro-Catabolic Cytokine for Chondrocytes

3.1 ABSTRACT

In osteoarthritis (OA), low-grade joint inflammation promotes altered chondrocyte differentiation and cartilage catabolism. S100/calgranulins share conserved calcium-binding EF-hand domains, associate noncovalently as homodimers and heterodimers, and are secreted and bind Receptor for Advanced Glycation End-products (RAGE). Chondrocyte RAGE expression and S100A11 release are stimulated by IL-1β in vitro and increase in OA cartilage *in situ*. Exogenous S100A11 stimulates chondrocyte hypertrophic differentiation. Moreover, S100A11 is covalently crosslinked by transamidation catalyzed by transglutaminase 2 (TG2), itself an inflammation-regulated and redox stress-inducible mediator of chondrocyte hypertrophic differentiation. Here, we studied mouse femoral head articular cartilage explants and knee chondrocytes, and a soluble recombinant double point mutant (K3R/Q102N) of S100A11 TG2 transamidation substrate sites. Both TG2 and RAGE knockout cartilage explants retained IL-1β responsiveness. The K3R/Q102N mutant of S100A11 retained the capacity to bind to RAGE and chondrocytes but lost the capacity to signal via the p38 MAPK pathway or induce chondrocyte hypertrophy and glycosaminoglycans (GAG) release. S100A11 failed to induce hypertrophy, glycosaminoglycans (GAG) release, and appearance of the aggrecanase neoepitope NITEGE in both RAGE and TG2 knockout cartilages. We conclude that transamidation by TG2 transforms S100A11 into a covalently bonded homodimer that acquires the capacity to signal through the p38 MAPK pathway, accelerate chondrocyte hypertrophy and matrix catabolism, and thereby couple inflammation with chondrocyte activation to potentially promote OA progression.

3.2 INTRODUCTION

Maintenance of the structural and functional integrity of articular cartilage requires a balance between anabolic and catabolic activities of chondrocytes, the sole cells in hyaline articular cartilage [152]. In osteoarthritis (OA), low-grade inflammation, associated with increased chondrocyte IL-1β expression, develops within cartilage and variably in synovium [153, 154]. IL-1β promotes chondrocyte de-differentiation, and stimulates oxidative stress and matrix catabolism modulated partly by induction of iNOS, MMP-13 and ADAMTS-5 [152, 155]. Certain other conventional inflammatory cytokines up-regulated in OA cartilage *in situ* (TNFα [42] and the chemokines CXCL1 and CXCL8 [52]) induce chondrocyte maturation to hypertrophy [57, 156], a state intimately linked in growth plate physiology to a shift in collagen synthesis from type II to type X, as well as other changes in extracellular matrix organization and an increased capacity to calcify [157, 158]. Significantly, evidence of chondrocyte hypertrophic differentiation typically appears within OA cartilage chondrocyte populations in the course of OA [23, 28].

Inhibition of IL-1β only partially inhibits experimental OA [29, 31, 159], and thus the respective roles of other inflammatory mediators in OA remain to be defined. S100/calgranulins are a family of more than 20 proteins of ~10-12 kDa that share conserved calcium-binding EF-hand domains and associate noncovalently as homodimers and heterodimers [102]. Inside cells, the major functions of S100/calgranulins appear to be mediated by their capacity to shuttle calcium between subcellular compartments [102]. When released by cells, some S100/calgranulins can act as unconventional inflammatory

cytokines (e.g., phagocyte-derived S100A8 and S100A9) [160, 161], and S100B, S100A4, and S100A11 can activate cultured chondrocytes [91, 162, 163]. S100/calgranulins signal in part by binding the Receptor for Advanced Glycation End-products (RAGE), a member of the immunoglobulin superfamily [79]. RAGE, a patterning receptor for at least four classes of ligands, does not scavenge ligands by internalization, but instead can promote activation of NF-κB, JAK-STAT and MAPK signaling dependent on the RAGE cytosolic tail domain [79]. IL-1β increases RAGE expression in cultured chondrocytes, and RAGE expression, and S100A11 surrounding chondrocytes, are elevated in OA cartilage in situ [91, 162]. In cultured chondrocytes, IL-1β, TNFα, and CXCL8 induce S100A11 release. Moreover, hypertrophic chondrocyte differentiation induced by exogenous S100A11, CXCL8 and TNFα, are dependent on RAGE signaling transduced in part by the p38 MAPK pathway [91, 162].

Clustering of RAGE by multimeric S100/calgranulins appears to enhance RAGE-dependent signal transduction [123-125]. Significantly, transglutaminase 2 (TG2) stimulates covalent S100A11 homodimerization by catalyzing formation of N-epsilon (gamma-glutamyl) lysyl isopeptide bonds [113, 122]. Moreover, cultured articular chondrocytes express TG2 [164], and TG catalytic activity and TG-catalyzed isopeptide bond formation are substantially increased in human knee OA cartilage chondrocytes [165, 166]. Furthermore, TG2 is the principal catalytically active TG isoenzyme in OA cartilage extracellular matrix in situ [167]. Significantly IL-1β, TNFα, CXCL1 and CXCL8 induce TG activity in cultured chondrocytes [57, 166], and TG2 release is essential for chemokine-induced chondrocyte hypertrophy [130, 167]. Hence, S100A11,

RAGE, and TG2 are expressed by chondrocytes, are modulated by inflammatory cytokines, and each can promote chondrocyte hypertrophic differentiation. Here, we observe that transamidation by TG2 transforms S100A11 into an unconventional inflammatory cytokine that, by acquiring the capacity to signal though RAGE, modulates chondrocyte differentiation and promotes matrix catabolism.

3.3 RESULTS

Differentiation and Function of TG2 and RAGE knockout cartilage explants and chondrocytes

We first isolated TG2 and RAGE knockout acetabular cartilage explants, which we observed to retain responsiveness to IL-1β comparable to that for wild type cartilage explants, as assessed by IL-1β-induced release of both GAG and NO (Figure 3.1). In addition, we isolated immature mouse knee articular chondrocytes, and verified, using RT-PCR and immunocytochemistry, expression of the chondrocyte-specific matrix proteins collagen type II and aggrecan, with only trace expression of collagen I (Figure 3.2A,B). In response to IL-1β, the wild type chondrocytes but not $TG2^{-/-}$ chondrocytes increased the level of TG catalytic activity in the conditioned media in association with secretion of TG2 (Figure 3.2C,D).

Next, we employed exogenous recombinant human TG2 K173L, a catalytically active TG2 mutant that lacks the capacity to bind GTP, and which we confirmed [119] to be insufficient by itself to induce chondrocyte hypertrophy. Exogenous S100A11 induces the stereotypic chondrocyte hypertrophy marker type X collagen at 4 days in cultured normal human chondrocytes [162]. Here, addition of TG2 K173L to murine knee chondrocytes rendered exogenous S100A11 able to induce type X collagen at 2 days in culture, a point at which S100A11 had not yet induced type X collagen (Figure 3.3A).

Previous studies of RAGE function in chondrocytes have not studied complete deficiency of RAGE by assessment of knockout animals [162]. Here, we first observed that S100A11 failed to induce type X collagen in $RAGE^{-/-}$ chondrocytes (Figure 3.3A). Second, we studied induction of type X collagen by immunohistochemistry of mouse hip

cartilage explants, bearing in mind the limitations that normal mouse knee articular cartilage does demonstrate some type X collagen expression in situ, and that subchondral bone in such specimens normally bears hypertrophic chondrocytes [168]. We observed that RAGE knockout blunted the capacity of S100A11 to induce type X collagen in the articular cartilage zone of explants (Figure 3.3B). Under these conditions, the capacity of S100A11 to induce type X collagen in the articular cartilage zone of the explants was markedly increased by concurrent addition of TG2 K173L (Figure 3.3B).

Effects of TG2 transamidation on the capacity of S100A11 to activate chondrocytes

We carried out charge-conservative point mutations of the described transamidation substrate sites of human S100A11 (K3 and Q102 to R3 and N102, respectively) (Figure 3.4A). Isolated recombinant human S100A11 K3R/Q102N, examined under non-reducing conditions was monomeric, whereas wild type S100A11 was found to be in monomeric and dimeric forms (Figure 3.4B). Upon incubation with 100 ng/ml TG2, and examination by SDS-PAGE and Western blotting under reducing conditions, we observed that TG2 stimulated covalent dimerization of wild type S100A11 but not S100A11 K3R/Q102N (Figure 3.4C). Since the S100A11 K3R/Q102N mutant did not naturally assemble into multimers, and since other S100/calgranulins can oligomerize via formation of disulfide bonds [169], we tested if S100A11 K3R/Q102N was able to form disulfide-bonded multimers. We observed that copper oxidation of S100A11 K3R/Q102N induced assembly into dimers and tetramers sensitive to reduction of the disulfide bonds with β-mercaptoethanol (Figure 3.4D).

Using an ELISA system to assess S100A11 binding to human chondrocytic CH-8 cells, we observed that both wild type S100A11 and K3R/Q102N mutant S100A11 were able to bind the chondrocytic cells in a RAGE-dependent manner (Figure 3.4E). This was assayed using a goat polyclonal blocking antibody that recognized RAGE with high selectivity in the CH-8 human chondrocytic cells (Figure 3.4F). Since we previously established critical linkage between MKK3 signaling and its kinase substrate p38 MAPK in the hypertrophic differentiation response to S100A11 [162], we next assessed the capacity of the S100A11 K3R/Q102N mutant to signal (Figure 3.5). For these signaling studies, we employed the CH-8 cells, since we observed CH-8 cells to express significantly less extracellular basal transamidation specific activity than normal human articular chondrocytes (data not shown). We demonstrated that antibody-induced cross-linking of RAGE induced p38 phosphorylation, but that the S100A11 K3R/Q102N mutation eliminated the capacity of S100A11 to induce detectable p38 phosphorylation in chondrocytic CH-8 cells (Figure 3.5A, B). We noted that S100A11 induced detectable p38 phosphorylation at 45 minutes but not at earlier tested time points in CH-8 cells (Figure 3.5A,C), whereas wild type S100A11 pretreated with the catalytically active K173L TG2 mutant induced p38 phosphorylation by 5 minutes in CH-8 cells (Figure 3.5C). In contrast, both the copper-oxidized wild type S100A11, and the K3R/Q102N S100A11 induced to multimerize (via disulfide bond formation) in response to copper oxidation (Figure 3.4D), failed to induce the rapid p38 phosphorylation response in CH-8 cells (Figure 3.5C).

At 4 days in culture, we detected S100A11-induced expression of type X collagen in $TG2^{+/+}$ chondrocytes and cartilage explants. In contrast, S100A11 K3R/Q102N failed

to induce type X collagen in wild type chondrocytes and cartilage explants (Figure 3.6A, B). As a control, S100A11 bearing a distinct charge-conservative lysine mutation K52R was able to form covalent dimers and to stimulate type X collagen expression (data not shown).

Wild type S100A11 failed to induce type X collagen in $TG2^{-/-}$ chondrocytes and cartilage explants (Figure 3.6A, B). To rule out that adaptive effects of germline TG2 deficiency were responsible for modulating the effects of S100A11 on chondrocytes, we knocked down TG2 in CH-8 cells by RNA interference using short hairpin and scrambled control sequences for TG2. Efficiency of TG2 knockdown by shTG2 was validated at the level of TG2 protein expression showing a >90% decrease in TG2 (Figure 3.6C). TG2 knockdown rendered S100A11 unable to induce type X collagen (Figure 3.6D). S100A11-induced type X collagen expression was rescued in cells subjected to TG2 knockdown by addition of the human TG2 K173L mutant (Figure 3.6D).

TG2 is required to transform S100A11 into an inducer of cartilage matrix catabolism.

We observed a 2-fold induction of GAG release in response to S100A11 in wild type ($RAGE^{+/+}$) cartilage explants (Figure 3.7A), an effect comparable to that of IL-1β (Figure 3.1A), and which was not observed in $RAGE^{-/-}$ cartilage explants (Figure 3.7A). S100A11 induced the aggrecanase neoepitope NITEGE in the $RAGE^{+/+}$ explants, a response lacking in $RAGE^{-/-}$ cartilage explants (Figure 3.7B). S100A11 K3R/Q102N failed to induce GAG release and the aggrecanase neoepitope in wild type cartilage explants (Figure 3.8A and C), and copper oxidation of K3R/Q102N S100A11 did not render it able to induce GAG release in the $TG2^{-/-}$ cartilage explants (data not shown).

Last, failure of S100A11 to induce GAG release and the aggrecanase neoepitope NITEGE in $TG2^{-/-}$ explants demonstrated correction by addition of catalytically active but not catalytically inactive TG2 to the $TG2^{-/-}$ cartilage explants (Figure 3.8 B and C).

3.4 DISCUSSION

Increased chondrocyte expression of pro-inflammatory cytokines such as IL-1β and TNFα mediate articular cartilage responses to injury in OA through effects on chondrocyte differentiation, as well as shifts from matrix anabolic to catabolic gene expression programs [152]. Additional regulatory loops that translate inflammation into cartilage responses to injury in OA include the conversion of inflammatory cytokines to active, secreted forms, as catalyzed by proteases themselves subject to regulation by endoproteolysis (e.g., caspase-1 for IL-1β, and TACE for TNFα).

The capacity of transamidation by TG2 to regulate activation of latent TGFβ [170] has the potential to impact on regulation of inflammatory responses within OA cartilage [57]. The primary rationale for the current study of functional linkage between TG2 and S100A11 was based on the observations that multimerization of secreted S100A11 in cultured human chondrocytes occurs in response to CXCL8 [162], and that TG2 plays a major role in mediating CXCL8-induced chondrocyte hypertrophic differentiation [57]. Here, we observed a novel TG2 function in chondrocytes, whereby transamidation by TG2 of S100A11 converted the secreted calgranulin into an inflammatory cytokine-like promoter of chondrocyte hypertrophy and matrix catabolism.

TG2 multifunctionality includes GTPase and fibronectin binding activities [167], but complementary lines of evidence demonstrated that TG2 transamidation catalytic activity was critical for S100A11 to induce chondrocyte hypertrophy and cartilage matrix catabolism. First, the combination of exogenous recombinant S100A11 and the catalytically active but GTP binding site functional mutant TG2 K173L accelerated

chondrocyte hypertrophy. Second, the dual TG2 transamidation substrate site mutant S100A11 K3R/Q102N, which bound to chondrocytes in a RAGE-dependent manner, lost the capacity to induce p38 phosphorylation or hypertrophy, or to suppress proteoglycans synthesis in chondrocytes, and failed to induce the aggrecanase neoepitope NITEGE or GAG release in cartilage explants. Third, S100A11 failed to induce chondrocyte hypertrophy, or to stimulate the aggrecanase neoepitope NITEGE and GAG release in TG2 knockout chondrocytes or cartilage explants.

S100/calgranulins constitutively exist as anti-parallel, noncovalently associated homodimers, heterodimers and other multimers [102]. S100/calgranulin dimer formation is mediated by calcium binding and the interaction of hydrophobic globular domains derived from helices I and IV from each monomer [171]. TG-dependent modification may inactivate certain S100/calgranulin functions by conformational change [122]. However, S100/calgranulin multimerization is thought to enhance RAGE-mediated intracellular signaling cascades at least in part by promoting ligand-induced RAGE oligomerization [123-125]. Significantly, there is increasing evidence of receptor oligomerization as a general mechanism for signal propagation by the closely related cytokine family of receptors [172-174]. Notably, in this study, the K3R/Q102N S100A11 mutant that lost the capacity to oligomerize also failed to induce chondrocyte hypertrophy, GAG release, and the aggrecanase neoepitope NITEGE.

S100A11 has been defined to be a substrate for TG2-dependent covalent crosslinking via two transamidation substrate domains (at the N-terminal K3 in alpha helix I and the C-terminal Q102 domain in alpha helix IV [122]. TGs generally catalyze transamidation in a manner highly selective for glutamine acyl donor substrate motifs

[122], with the configuration of the amino acids surrounding the lysine residue being of less importance for formation of N-epsilon (gamma-glutamyl) lysyl isopeptide bonds [175]. Though it is not yet clear how TGs differentially recognize and interact with individual glutamines and lysines and their surrounding amino acids, TGs preferentially modify highly accessible glutamine and lysine residues such as in the relatively exposed N-termini and C-termini of S100A11, and also in S100A10, the calgranulin most closely related to S100A11 [122]. It is possible that other chondrocyte-expressed S100/calgranulins could also be TG2 substrates. One example is S100A4, which forms multimers [124, 176], bears highly exposed C-terminal glutamine and lysine residues, stimulates MMP-13 expression in cultured chondrocytes dependent on RAGE [163], and promotes the expression of a variety of MMPs in cells other than chondrocytes [177].

Functional characteristics of multimeric S100 proteins linked by disulfide bonds or noncovalent associations have been studied most extensively in cells other than chondrocytes. In this context, S100B dimers linked together by disulfide bonds induce neurite outgrowth, but dimers without disulfide bonds can induce an inflammatory response in glial cells [178, 179]. Furthermore, noncovalent tetramers of S100B found in human brain extracts appear to induce RAGE multimerization [180]. Additionally, the Ca^{2+}-dependant S100A8/A9 tetramer promotes the formation of microtubules [181] and the S100A12 hexamer associates with RAGE [182]. Since TG2-induced isopeptide bond formation was critical for S100A11 pro-inflammatory signaling in chondrocytes, but disulfide-bonded S100A11 multimers generated by copper oxidation did not share this activity, the mode in which S100A11 is multimerized and its consequent conformation appear essential for pro-inflammatory effects in chondrocytes.

Limitations of this study include lack of kinetic binding studies and assessments of ligand-induced RAGE multimerization in chondrocytes. We did not perform circular dichroism studies for analyses of folding of S100A11 in the presence of TG2 or for possibly altered folding for the S100A11 K3R/Q102N mutant. Glasson et al have described a critical role for ADAMTS-5-driven aggrecanolysis in a murine instability OA model [155]. Here, we observed that S100A11 induced both the aggrecanase neoepitope NITEGE and GAG release in cartilage explants, but we did not define the net role in matrix catabolism of ADAMTS-5 activation, relative to potential release and activation of ADAMTS-4 or MMP-3, for example. Moreover, the mechanism of the induction by S100A11 of the aggrecanase neoepitope was beyond the scope of this study. We have observed that S100A11 does not induce ADAMTS-5 transcription in chondrocytes but does increase chondrocyte secretion of ADAMTS-5 protein (D.Cecil, unpublished observation). Regulation of ADAMTS-5 catalytic activity is complex, as it is not simply controlled by furin-induced endoproteolysis [183]. S100A11 could theoretically act indirectly by regulating factors that mediate increased ADAMTS-5 catalytic activity. Though TG2 knockout blunted pro-catabolic responses of chondrocytes and cartilages to S100A11, we did not examine potential effects on S100A11 structure and function exerted by the other major chondrocyte-expressed TG isoenzyme FXIIIA [164]. Last, we have detected multimers of S100A11 in extracts of human OA cartilage that retain multimerization under reducing conditions in SDS-PAGE (D. Cecil, unpublished observation). However, we have not quantitatively analyzed differences in S100A11 multimerization in OA relative to normal cartilage. Moreover, we have not yet directly

assessed the impact of knockout of S100A11, RAGE, or TG2 on experimental OA in vivo.

TG2 release is critical for determining the phenotype of healing of multiple forms of tissue injury [113]. This study reveals that post-translational modification of S100A11 by TG2 stimulates chondrocyte hypertrophic differentiation and transforms S100A11 into a pro-catabolic inflammatory cytokine-like molecule for cartilage via RAGE signaling. Therefore, S100A11, subject to transformation by TG2, is able to function as an unconventional inflammatory mediator of altered chondrocyte differentiation and matrix remodeling, analogous to functions of proteolytically derived fibronectin fragments that also are increased within OA cartilages [126]. S100A11 release by chondrocytes is regulated partly by conventional cytokines. Nevertheless, the findings of this study, including the retention of responsiveness to IL-1β by RAGE and TG2 knockout mouse cartilage explants, reveal S100A11 to be capable of independently inducing alterations in chondrocyte differentiation and extracellular matrix organization. Hence, S100A11 and TG2 have a unique functional linkage with the potential to translate low-grade cartilage inflammation into the progression of OA.

The text of chapter 3 is a reprint of the material as it appears in the Journal of Immunology, Cecil DL, Terkeltaub R. “Transamidation by Transglutaminase 2 Transforms S100A11 Calgranulin into a Pro-Catabolic Cytokine for Chondrocytes”, 180(12):8378-85, 2008. I was the primary researcher and author and the co-author listed in this publication directed and supervised the research which forms the basis of this chapter.

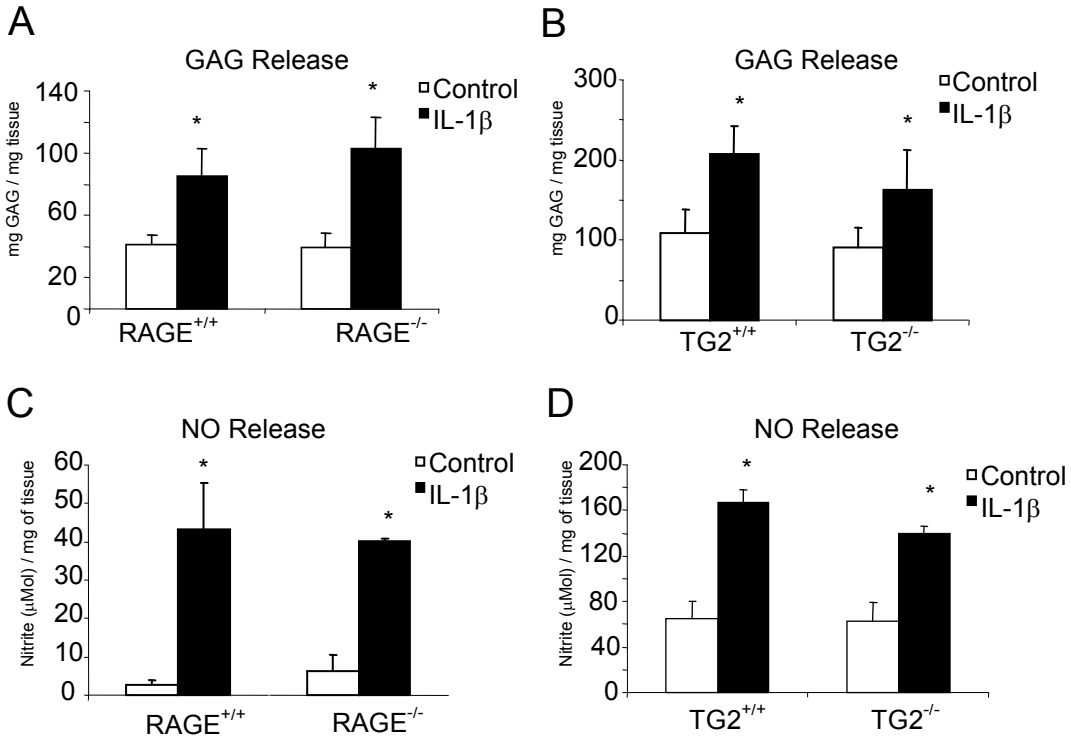


FIGURE 3.1

Both RAGE and TG2 knockout mouse cartilage explants retain responsiveness to IL-1β. Mouse femoral head cartilage was isolated as described in the Methods. IL-1β (10 ng/ml) was added and GAG release was measured at 48 hours (A) and (B) and NO generation at 24 hours assayed using the Griess reaction (C) and (D). These data are representative of 3 separate experiments. *$p<0.05$ compared to unstimulated cells.

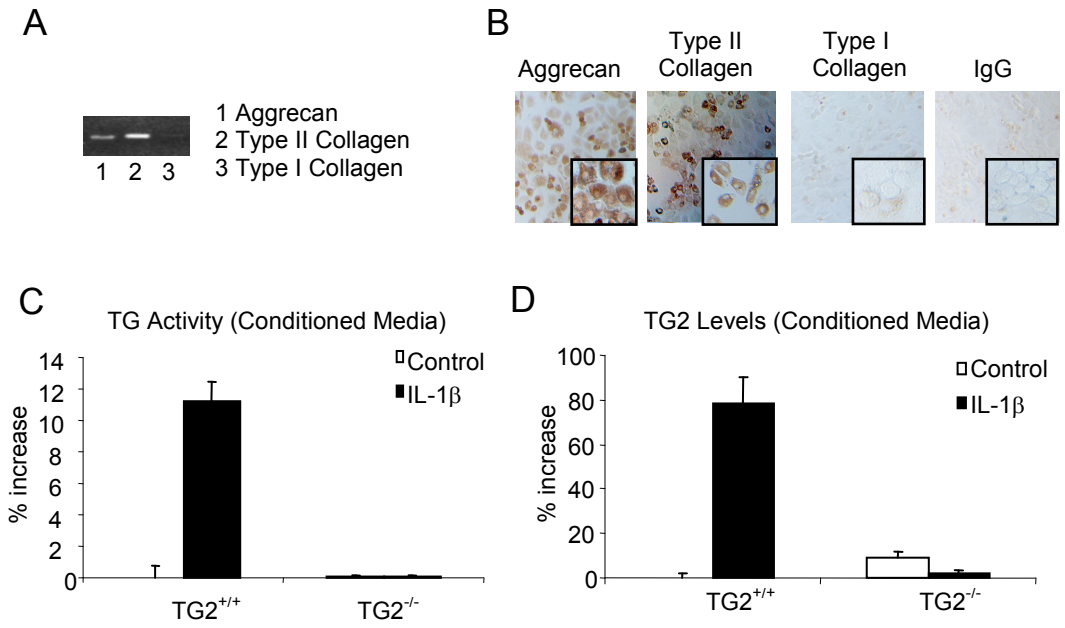


FIGURE 3.2

Characterization of extracellular matrix molecule expression and TG2 release in mouse articular chondrocytes. Articular chondrocytes from 7-8 day old mice were isolated as described in the Methods, and after 3 days in culture, (A) RT-PCR analysis and (B) immunocytochemistry (visualized at 40X (inset 100X)) were performed for collagen types II and I, and for aggrecan, with results representative of 5 different donors. Mouse chondrocytes were stimulated with 10 ng/ml IL-1β for 2 days, and (C) released TG catalytic activity was measured in the conditioned media, with results shown as % increase above the TG activity in unstimulated cells. (D) TG2 was measured by ELISA in the conditioned media, with results representing % increase above TG2 released by unstimulated cells. Data pooled from 4 experiments performed in triplicate. *$p<0.05$

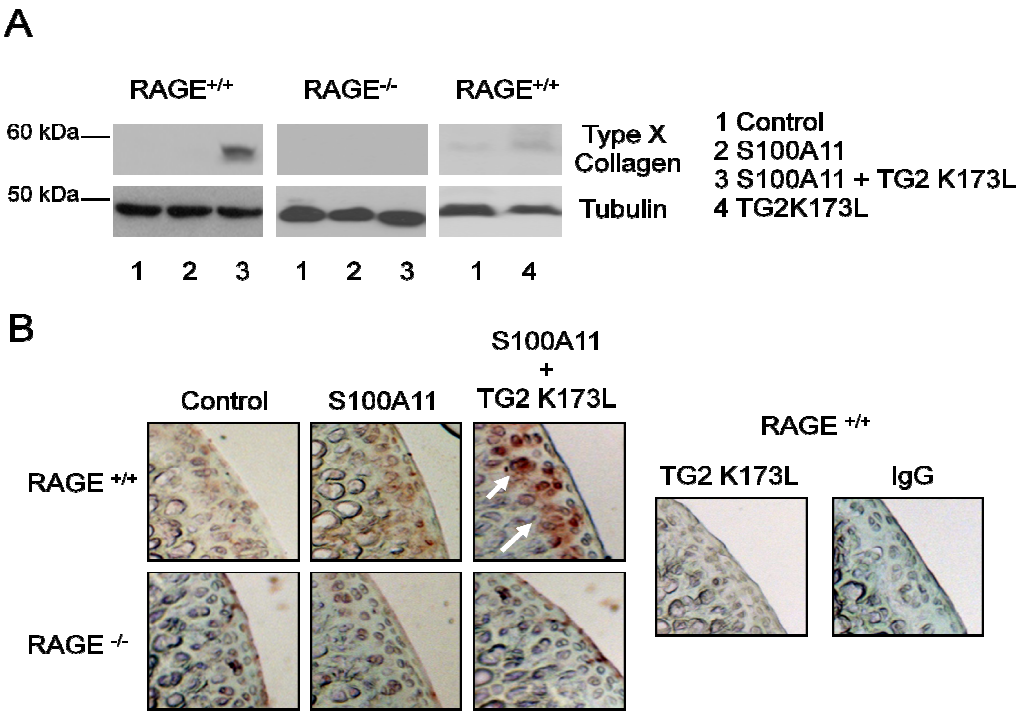


FIGURE 3.3
Exogenous TG2 accelerates S100A11-induced type X collagen expression in cultured chondrocytes and cartilage in organ culture. (A) After 3 days in culture that immediately followed isolation, mouse chondrocytes were treated with 100 ng/ml S100A11 with or without 100 ng/ml TG2 K173L. SDS-PAGE and Western blotting on cell lysates were performed for type X collagen after 2 days in culture. As a control, the effects on expression of type X collagen of the catalytically active but GTP-binding site functional mutant TG2 K173L were examined. (B) Mouse femoral head cartilage was treated as in (A). Frozen sections were examined by IHC for type X collagen as described in the Methods. Data representative of 5 different mouse donors.

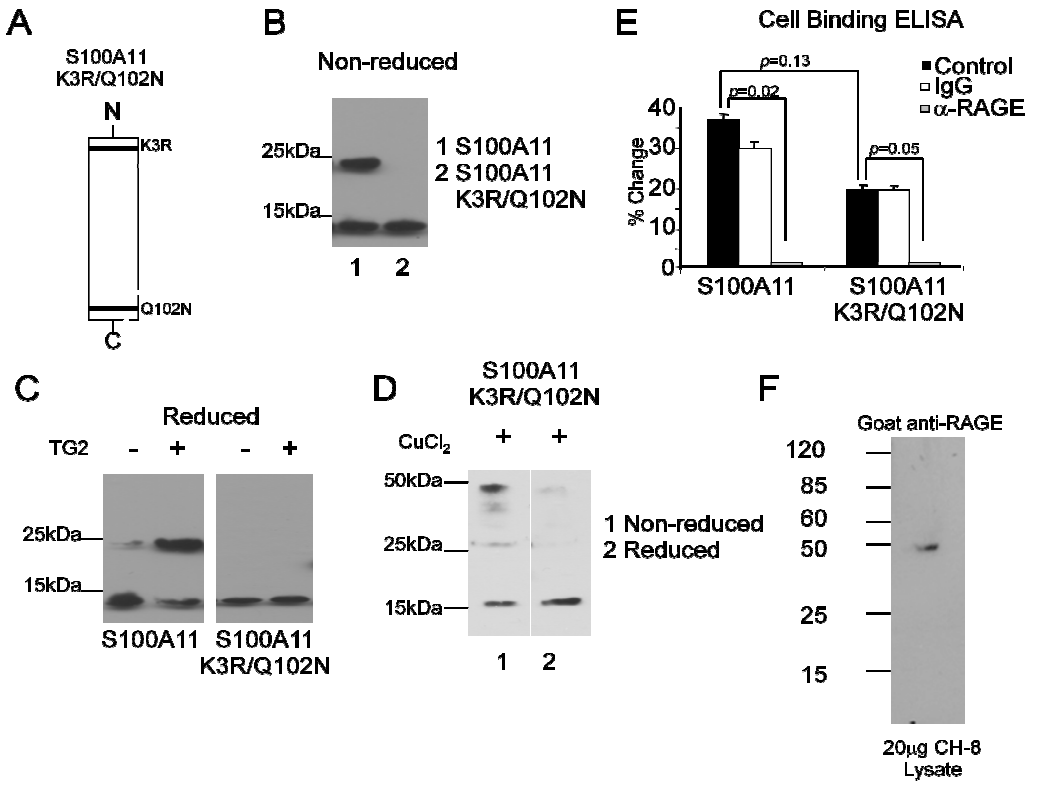


FIGURE 3.4

Characterization of the S100A11 K3R/Q102N mutant. (A) Graphic representation of the TG2 transamidation substrate sites of K3R and Q102N in the S100A11 protein. (B) Both TG2 transamidation sites in recombinant human S100A11 were altered by site-directed mutagenesis to generate S100A11 K3R/Q102N. After purification, S100A11 and S100A11 K3R/Q102N proteins were isolated. S100A11 was separated by SDS-PAGE under non-reducing conditions and Western blotting performed. The wild type S100A11 was detected as both monomeric (~ 12 kDa) and dimeric (~24 kDa), but the TG double transamidation site mutant was only detected in monomeric form. (C) 1 μg S100A11 and S100A11 K3R/Q102N were incubated with 100 ng/ml TG2 for 2 hours at 37°C, and SDS-PAGE under reducing conditions and Western blotting for S100A11 were performed. Data are representative of 4 different experiments. (D) 1 μg of S100A11 K3R/Q102N was incubated with 6 μM $CuCl_2$ for 1 hour at 37°C. SDS-PAGE under non-reducing and reducing conditions and Western blotting were performed for S100A11. Data are representative of 3 different experiments. (E) Cell-associated RAGE binding ELISA was performed as described in the Methods. The results are presented as % change compared to wells not receiving S100A11 or S100A11 K3R/Q102N, with data pooled from 4 experiments. (F) 20 μg of human chondrocytic CH-8 cell lysates was separated by SDS-PAGE under reducing conditions and Western blotting performed for RAGE using goat polyclonal antibodies.

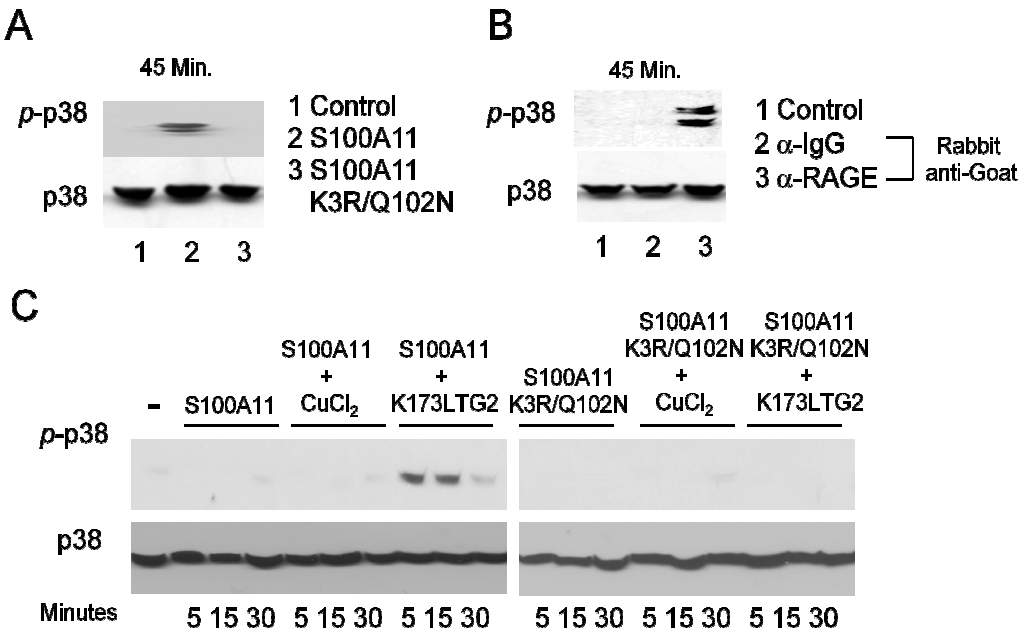


FIGURE 3.5

Decreased signaling capacity of the S100A11 K3R/Q102N mutant in human chondrocytic CH-8 cells. (A) CH-8 cells were treated with 100 ng/ml S100A11 or 100 ng/ml S100A11 K3R/Q102N for 45 min. SDS-PAGE/Western blotting of cell lysates was performed to assess p38 phosphorylation. Total p38 was visualized as a loading control. Data representative of 5 different experiments. (B) CH-8 cells were pretreated with anti-RAGE or control IgG. After several washes, 10 μg/ml rabbit anti-goat antibody was added for 45 min. SDS-PAGE/Western blotting of cell lysates was performed for p38 phosphorylation. Data representative of 4 experiments. (C) S100A11 and S100A11 K3R/Q102N were incubated with 100 ng/ml TG2 for 2 hours at 37°C or 6 μM $CuCl_2$ for 1 hour at 37°C, and then CH-8 cells were stimulated with 100 ng/ml S100A11, 100 ng/ml S100A11 K3R/Q102N, 100 ng/ml of TG2-pretreated calgranulins or 100 ng/ml $CuCl_2$ treated calgranulins for the times indicated. SDS-PAGE/Western blotting on cell lysates was performed for p38 phosphorylation and total p38, with data representative of 3 different experiments.

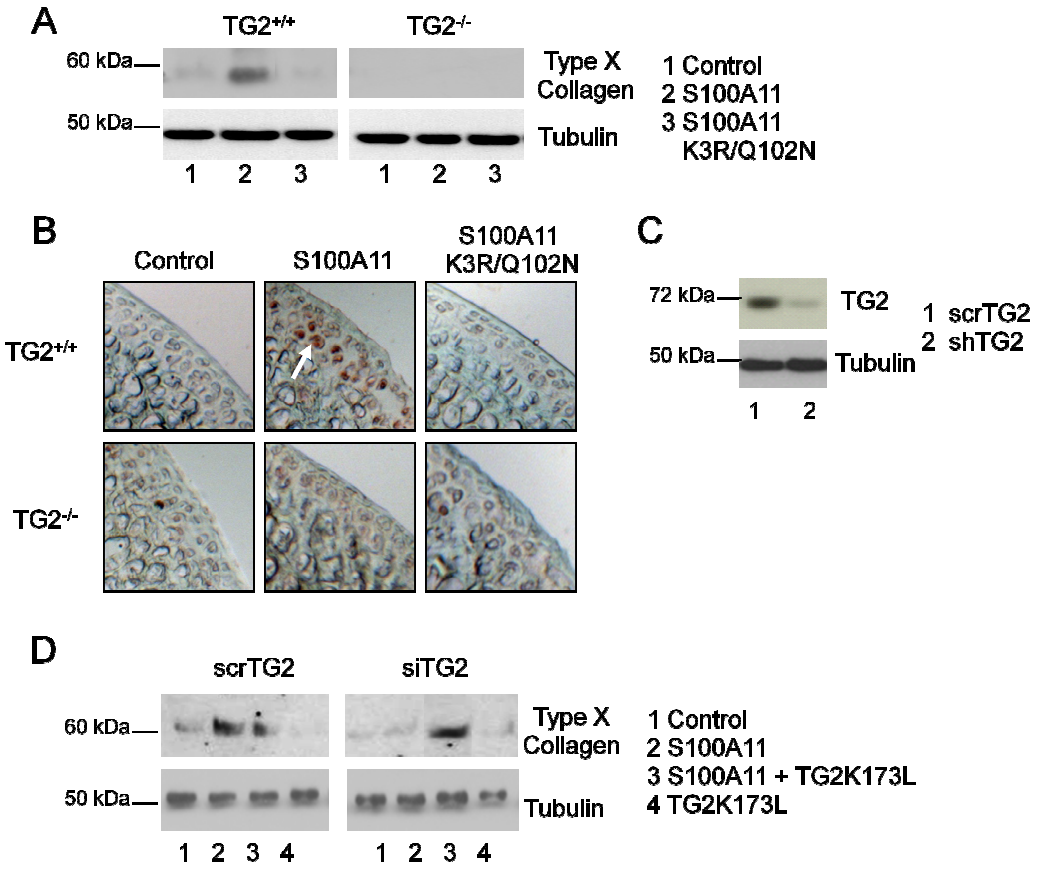


FIGURE 3.6

TG2 is required for S100A11 to induce chondrocyte hypertrophy. Mouse chondrocytes (0.1 X 10^6 cells/12-well dish) (A) or femoral head cartilage explants (B) of the indicated genotypes ($TG2^{-/-}$ and wild type controls) were stimulated with 100 ng/ml S100A11 or 100 ng/ml S100A11 K3R/Q102N. SDS-PAGE/Western blotting on cell lysates was performed for type X collagen after 4 days in culture. Frozen sections were examined by IHC for type X collagen as described in the Methods. Data representative of 7 different mouse donors. (C) For TG2 mRNA knockdown, CH-8 cells (0.25 x 10^6 cells/6-well dish) were transfected by lipofection with plasmid constructs encoding short hairpin TG2 (shTG2) or the control scrambled TG2 (scrTG2), as described in the Methods. TG2 expression in CH-8 cells transfected with scrTG2 versus shTG2, assessed by SDS-PAGE/Western blotting and densitometric analysis of cell lysates, verified >90% knockdown by shTG2. (D) After transfection, cells were transferred to 96-well polyHEME coated plates and 100 ng/ml S100A11 was added with or without 100 ng/mL TG2 K173L. SDS-PAGE/Western blotting on cell lysates was performed for type X collagen after 4 days in culture. Results representative of 3 experiments.

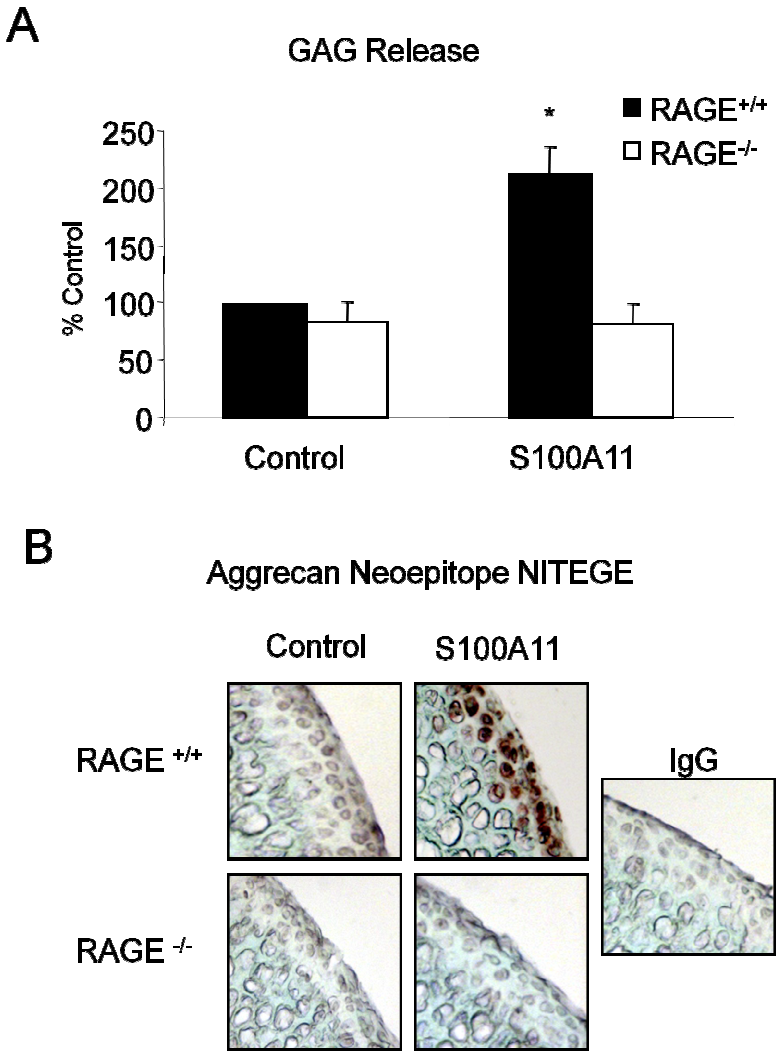


FIGURE 3.7
S100A11 stimulates GAG release in articular cartilage explants. Mouse femoral head cartilage explants were treated with 100 ng/ml S100A11. (A) At 48 hours, GAG release was measured in the conditioned media, with results indicated as % control (with S100A11 inducing 65.5 $\pm$ 9.3 mg GAG release / mg tissue in wild type cartilage explants). Data pooled from 11 different mouse donors of each genotype in replicates of 3. (B) Frozen sections were analyzed by IHC for the aggrecanase neoepitope NITEGE at 72 hours in culture. Data representative of 5 different mouse donors of each genotype. .*$p<0.05$ compared to unstimulated cells.

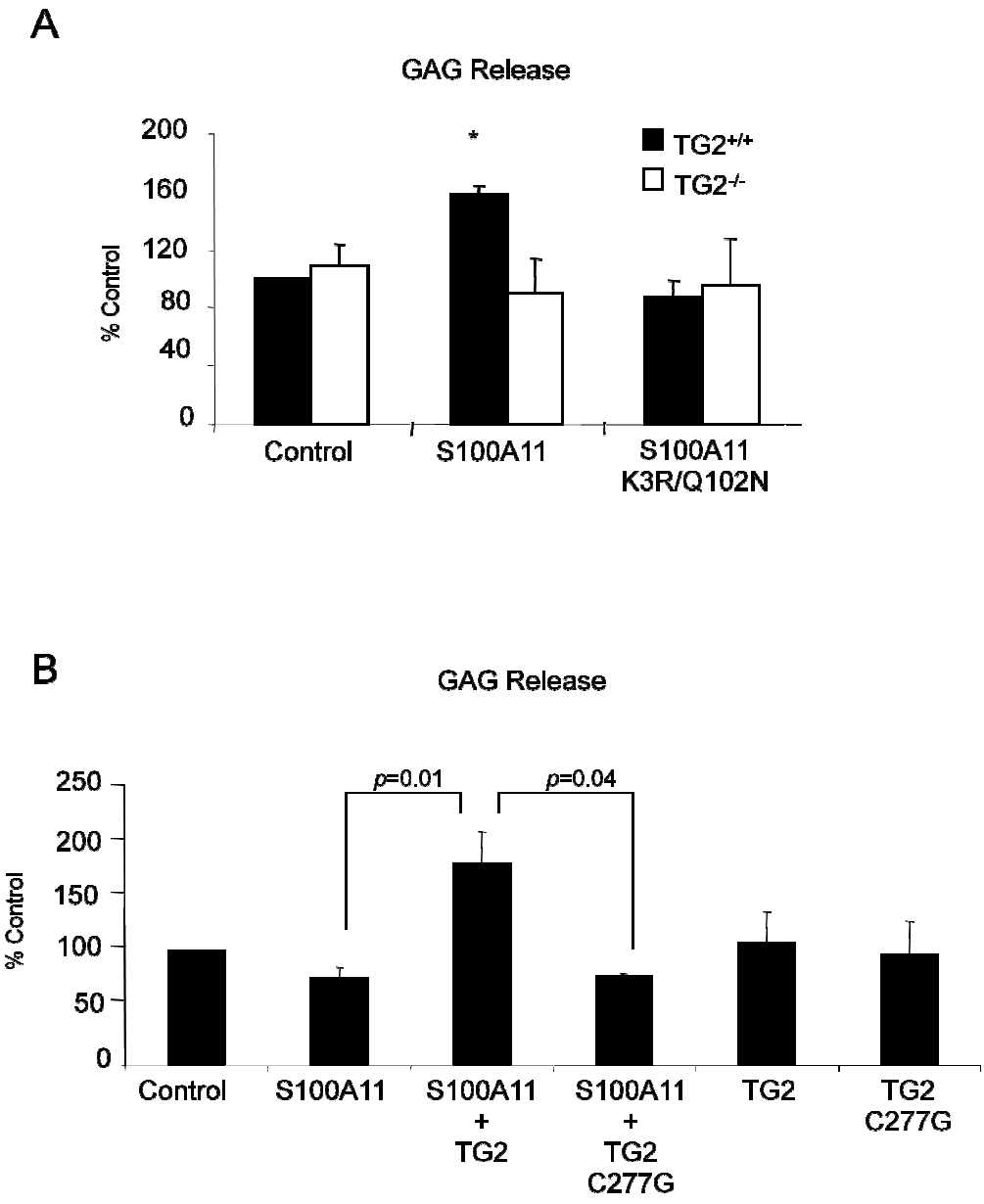


FIGURE 3.8
TG2 mediates the capacity of S100A11 to promote cartilage matrix catabolism. (A) $TG2^{+/+}$ and $TG2^{-/-}$ mouse femoral head cartilage explants were treated with 100 ng/ml S100A11 or 100 ng/ml S100A11 K3R/Q102N. At 48 hours, GAG release was measured in the conditioned media. Data pooled from 15 different mouse donors in replicates of 3. (B) $TG2^{-/-}$ mouse femoral head cartilage was treated with 100 ng/ml S100A11 with or without 100 ng/ml TG2 or 100 ng/ml of the catalytically inactive TG2 C277G mutant. After 2 days, GAG release was measured in the conditioned media. Data pooled from 8 different mouse donors studied in replicates of 3.

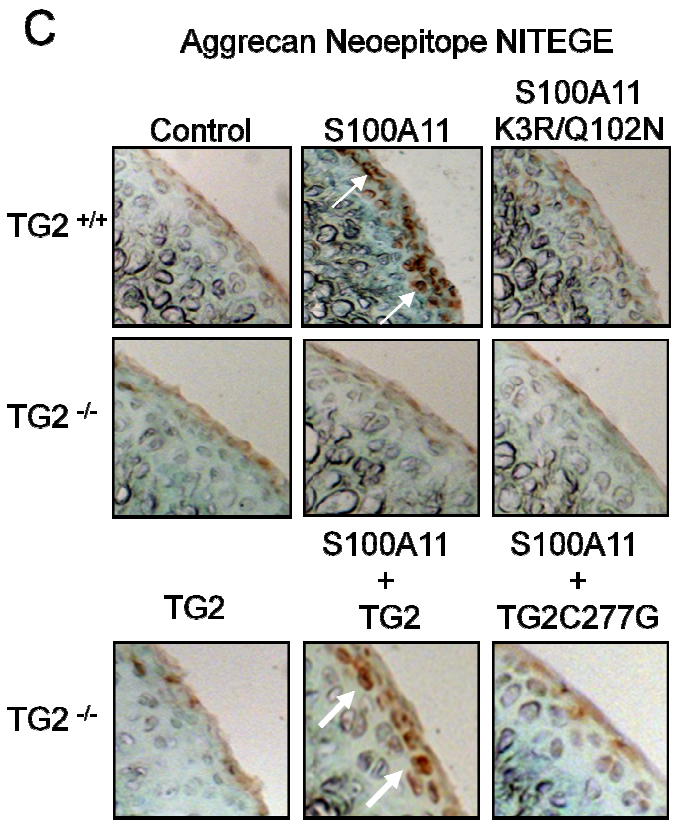


FIGURE 3.8 continued
TG2 mediates the capacity of S100A11 to promote cartilage matrix catabolism. (C) Frozen sections were analyzed by IHC for the aggrecanase neoepitope NITEGE at 72 hours in culture. Data representative of at least 4 different mouse donors of each genotype. *$p<0.05$ compared to unstimulated cells.

CHAPTER 4

RAGE Gene Deletion Fails to Protect Cartilage Degradation in a Murine Surgically-induced Instability Model of Osteoarthritis

4.1 ABSTRACT

Pathogenesis of osteoarthritis (OA) is modulated by chronic low-grade inflammation. *In vivo* models of chronic arterial, renal and neurological degenerative states associated with low-grade tissue inflammation exhibited less severe disease in mice lacking the Receptor for Advanced Glycation Endproducts ($RAGE^{-/-}$) and mice treated with a soluble form of RAGE (sRAGE). The calgranulin S100A11 has been implicated in OA progression by stimulating chondrocyte differentiation to hypertrophy and increasing proteoglycans (PG) depletion in a RAGE-dependent manner.

In this study, the role of RAGE in the pathogenesis of OA was examined *in vivo*. Instability was surgically induced in $RAGE^{-/-}$ and congenic wild-type controls with an anterior cruciate ligament tear (ACL-T) through a blind "stab" incision. The level of instability was monitored by proliferative changes in the marginal zones and ligaments. Eight weeks after surgery, cartilage degeneration, osteophyte formation and type X collagen expression significantly increased as instability increased, but there was no significant difference between $RAGE^{-/-}$ and congenic wild-type controls.

In conclusion, RAGE gene deletion does not protect cartilage from degeneration in the ACL-T model of OA. However, the function of RAGE has not been assessed in other models of surgically-induced OA. In addition, the roles of RAGE ligands, involvement of other receptors that recognize RAGE ligands and sRAGE have not been addressed.

4.2 INTRODUCTION

Osteoarthritis (OA) is not recognized as a classic inflammatory condition, however chronic, low-grade inflammation modulates pathogenesis. The synovial and cartilage-derived traditional cytokines IL-1β and TNFα, chemokines CXCL1 and CXCL8, and non-traditional cytokines S100/calgranulins contribute to OA progression [2, 31, 52, 162]. These inflammatory mediators participate in cartilage catabolism by inducing matrix-degrading proteases, including matrix metalloproteinases (MMPs) 1, 3 and 13, and ADAMTS-4 and -5 [2, 31, 52, 57, 126-129].

TNFα, CXCL1 and CXCL8 induce chondrocyte hypertrophic differentiation in the adult cartilage, recapitulating the chondrocyte maturation state in growth plate physiology [57, 162]. OA cartilages typically develop foci of hypertrophic chondrocytes, which contribute to the pathogenesis by decreasing type II collagen and aggrecan expression, increasing expression of type X collagen and MMP-13, and promoting pathologic calcification [23, 57, 130].

Recently, we demonstrated that secreted S100A11 induces type X collagen expression and cartilage catabolism through the Receptor for Advanced Glycation Endproducts (RAGE). RAGE, a member of the immunoglobulin superfamily, is a patterning receptor recognizing at least four different classes of ligands, including S100/calgranulins [83-86]. RAGE expression has been shown to increase in aging tissue, and recent evidence has associated RAGE expression with both age and the presence of OA pathology in humans [91].

RAGE has also been implicated in the pathogenesis of other chronic arterial, renal, and neurological degenerative states associated with low-grade tissue inflammation

[97-99, 184]. *In vitro* and *in vivo* blockade of RAGE by a truncated form of RAGE (sRAGE) reduced development of vascular disease and late complications of experimental diabetes, and prevented β-amyoid to cross the blood-brain barrier. An improvement in pathology was also observed in $RAGE^{-/-}$ mice, though to a lesser degree [94].

As RAGE appeared to be important to the pathology of other low-grade inflammatory conditions, we examined the role of this receptor in the pathogenesis and progression of OA. Surgically-induced instability models of OA are commonly used in dog, guinea pig, rabbit, rat, sheep and goat. In these models, the joint is larger and the architecture is easily manipulated [185, 186]. Recently, microsurgical techniques have been applied to the mouse knee joint where different combinations of ligament transection and meniscectomy results in varying severities of OA [77].

In this study, we transected the anterior cruciate ligament through a blind "stab" incision in $RAGE^{-/-}$ and congenic wild-type controls (Figure 4.1) [122]. This model results in progressive OA lesions that mimic naturally occurring trauma-induced human OA [187-189]. The severity of OA is directly correlated with the level of instability. The cartilage is marked by lesions that may occur in any location accompanied by superficial to middle zone chondrocyte and proteoglycans (PG) loss, and significant osteophyte formation [122].

4.3 RESULTS

RAGE null mice demonstrated similar joint scores, osteophyte size, PG loss, and type X collagen expression as congenic wild-type mice

There were no complications post-surgery and the mice resumed normal activity. Cartilage degeneration was not favored in a specific area of the joint and was observed in both medial and lateral tibial plateaus and femoral condyles (data not shown). Surgery was performed on both knee joints of all animals and the joints that had a 0 instability score were used as the sham controls.

To assess cartilage degeneration, scores were summed for both the tibial plateau and femoral condyle to arrive at the total joint score as described in the Methods (Figure 4.2). As the instability score increased, the total joint score significantly increased in both the wild type and RAGE null mice, but there was no significant difference in joint scores between the two genotypes. In both groups, as instability scores increased, the osteophyte size on the medial side also increased (Figure 4.3). There was negligible osteophyte formation observe on the lateral side, and no osteophyte generation was detected in the mice with instability scores of 0 or 1 (data not shown).

PG content and surface architecture were analyzed histologically by Toludine Blue staining (Figure 4.4). Joints with no instability score demonstrated significant PG content and normal cellularity. However, as the severity of instability increased there was a marked decrease in PG staining. With an instability score of 3, both the RAGE null and wild type cartilage demonstrated focal areas of complete chondrocyte loss and severe depletion of PG.

Normal mouse knee articular cartilage has been shown to express type X collagen *in situ* [168]. The articular cartilage in the sham control (instability score 0), demonstrated some type X collagen expression, but the expression was markedly upregulated in the instability score 3 joints (Figure 4.5). However, there was no difference in type X collagen expression levels between the $RAGE^{+/+}$ and $RAGE^{-/-}$ cartilage.

4.4 DISCUSSION

Spontaneous mouse models exhibiting OA with aging such as the STR/ort and C57BL/6 strains exhibit similar OA lesions to human disease [190, 191]. However, the progression of OA is slow, shows a high degree of variability and is dependent on genetic background [46]. Therefore, surgically-induced instability models have been employed to mimic the mechanisms of stress-induced OA [77].

In humans, anterior cruciate ligament tear (ACT-T) affects joint stability and can result in the development of OA after 10-15 years [192, 193]. The ACL-T model has been well characterized in dogs and rats, leading to progressive OA after 4 weeks [73, 75]. In this study, instability in the knee has been induced with a blind incision "stab" of the ACL. The "stab" ACL-T has several limitations: there can be variation in the transection of the ACL, with possible transection of the posterior cruciate ligament; insertion of the needle can result in subchondral bone damage; there can be unrestricted bleeding, and free blood in the joint space has been associated with increased risk of OA in patients with hemophilia [78, 194]. However, in this study, the severity of OA directly correlated with the degree of instability as measured by proliferative changes in the marginal zones and ligaments. In addition, the sham animals exhibited negligible degenerative changes, suggesting little iatrogenic effects or excessive bleeding.

It has been previously demonstrated that the calgranulin S100A11 stimulated type X collagen expression, GAG release and the aggrecan neoepitope NITEGE formation in a RAGE-dependent manner [195]. Therefore, one would expect the cartilage degeneration in the RAGE null joints would be significantly lower than that seen in the congenic wild-type controls after ACL-T. However, in this model where OA had

developed, RAGE null joints were not protected from cartilage damage. Glasson *et al.* have suggested that OA induced in the ACL-T model is still too severe to observe effects from gene knockouts or disease modifying OA drugs (DMOADs) [78]. Mice deficient in IL-1β, MMP-3, interleukin-1 converting enzyme and iNOS were not protected from OA induced by partial medial meniscectomy and medial collateral ligament transection. In fact more severe OA was observed in the contralateral cartilage of the IL-1β null mouse [44]. The IL-1β knockout joint was only protected from OA when instability was induced through the disruption of the medial meniscus (DMM) by transection of the medial meniscotibial ligament [46]. Therefore, it would be of interest to test the DMM model on the RAGE null mice.

The role of RAGE in modulating the progression of vascular disease and diabetic neuropathy/nephropathy has been evaluated in $RAGE^{-/-}$ mice and wild-type mice treated with sRAGE. Diabetic nephropathy, neuropathy and neointimal expansion was significantly increased in diabetic mice overexpressing RAGE in the vasculature, but was reduced in $RAGE^{-/-}$ mice. However, the wild-type mice treated with sRAGE demonstrated even less disease than that observed in the $RAGE^{-/-}$ mice [97-99, 184]. Additionally, RAGE gene deletion failed to protect in models of delayed hypersensitivity and autoimmune encephalomyelitis, but mice treated with sRAGE exhibited a significant reduction in disease pathology [84, 96].

These results suggest that RAGE itself plays a less significant role than its ligands. As RAGE is a multiligand receptor, the protection observed with sRAGE treatment might not only result from the sequestering of ligands from cell surface RAGE, but possibly affect the binding to other receptors or cell structures.

Advanced Glycation Endproducts (AGEs) accumulate in OA cartilage resulting in decreased proteoglycans and collagen synthesis *in vitro* [146], and has been shown to associate with CD36 and AGER-1 [196, 197]. HMGB-1 is expressed by articular chondrocytes [198], and secreted HMGB-1 can be recognized by TLR2 or TLR4 [199, 200]. RAGE-independent affects have been observed for S100B, S100A4, and S100A12 in cells other than chondrocytes [201], and it was recently demonstrated that TLR4 is the dominant receptor for S100A8/9 signaling in macrophages [202]. Significantly, S100B, S100A4 and S100A8/9 are all upregulated in OA cartilage [91, 163, 203]. In addition, TNFα- and CXCL8-stimulated chondrocyte hypertrophic differentiation was inhibited by sRAGE [162].

Finally, since the joint tissues completely lack RAGE, the endogenous and presumably therapeutic release of sRAGE can no longer aid in the preservation of cartilage degeneration. As it appears RAGE ligands have a greater effect on pathogenesis in other low-grade inflammatory conditions than RAGE itself, it would be of interest to treat wild-type mice with sRAGE after induction of OA.

In conclusion, RAGE gene deletion did not protect against cartilage degradation in the ACL-T model of OA. However, these results do not exclude a role for RAGE or RAGE ligands in the pathogenesis or progression of OA.

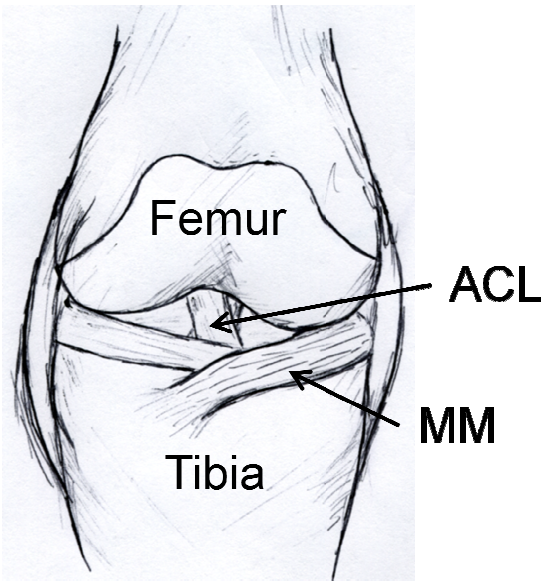


FIGURE 4.1
Diagram of the anterior view of right knee joint of the mouse. MM: medial meniscus; ACL: anterior cruciate ligament. The ACL is transected in the "stab" model of the ACL-T.

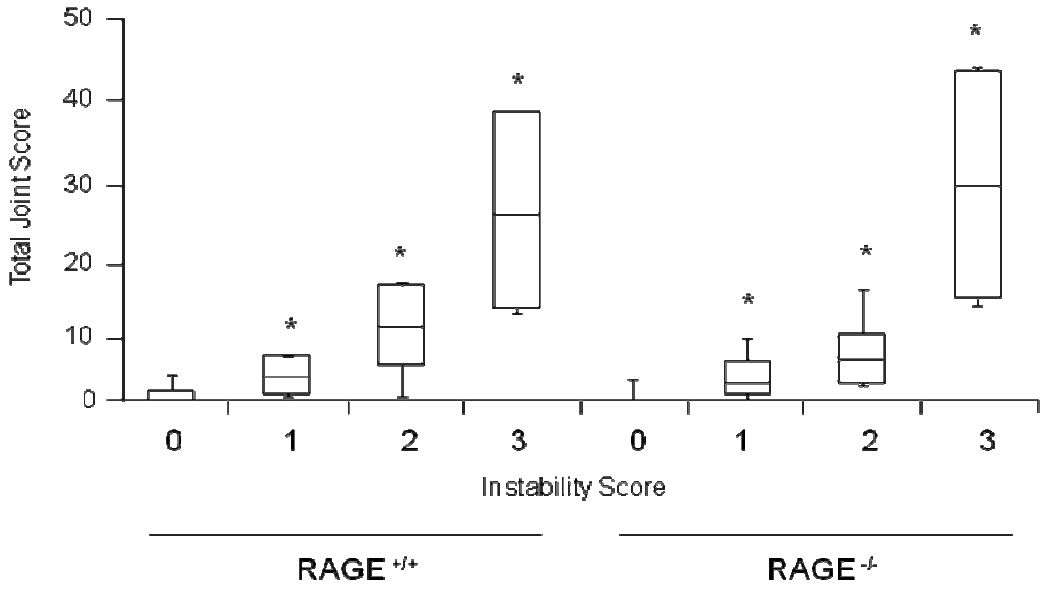


FIGURE 4.2
Similar joint scores observed in RAGE $^{+/+}$ and RAGE$^{-/-}$ knees after joint instability. The ACL of the RAGE$^{+/+}$ and RAGE$^{-/-}$ mice knees were "stabbed" with a 25 gauge needle as described in the Methods. After two months, the mice were sacrificed and the knee joints analyzed. The total joint score measures cartilage degeneration over the whole joint and is represented as box and whisker plots showing the twenty-fifth through seventy-fifth percentiles boxed and the median joint score as the central horizontal line. The data range is represented by whiskers. RAGE$^{+/+}$ with instability at 0 (n=50), 1 (n=7), 2 (n=4) and 3 (n=6) and RAGE$^{-/-}$ with instability scores of 0 (n=49), 1 (n=11), 2 (n=6) and 3 (n=3). * $p<0.01$ compared to 0 instability score.

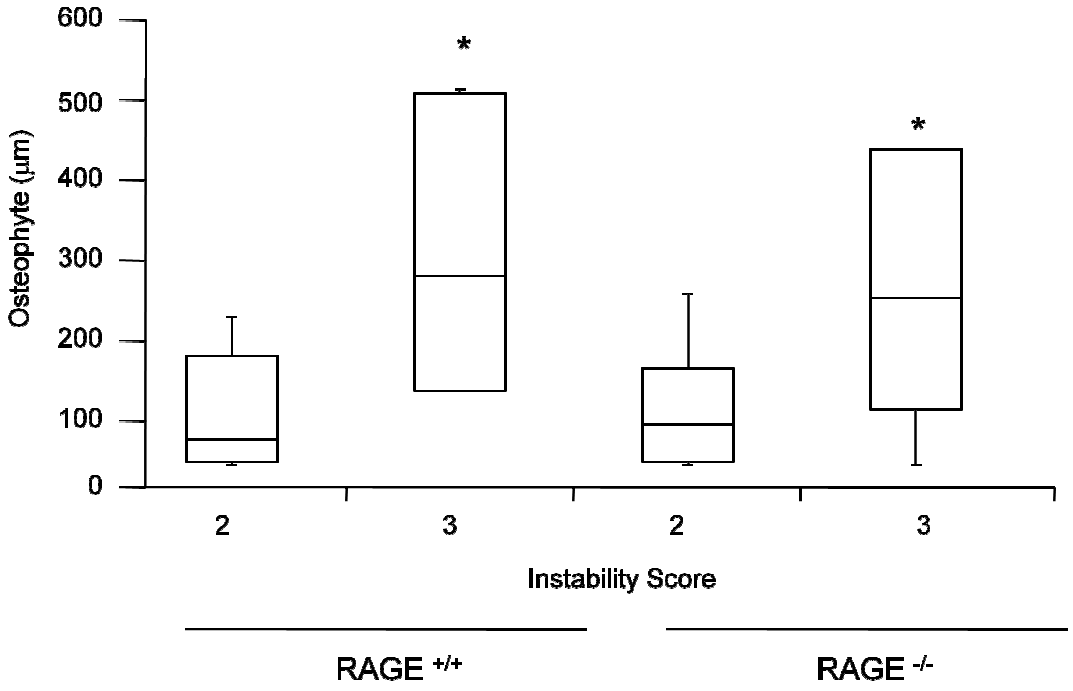


FIGURE 4.3
Similar size of medial osteophytes observed in RAGE $^{+/+}$ and RAGE$^{-/-}$ knees after joint instability. The osteophyte width is represented as box and whisker plots showing the twenty-fifth through seventy-fifth percentiles boxed and the median joint score as the central horizontal line. The data range is represented by whiskers. *$p<0.01$ compared to instability score 2.

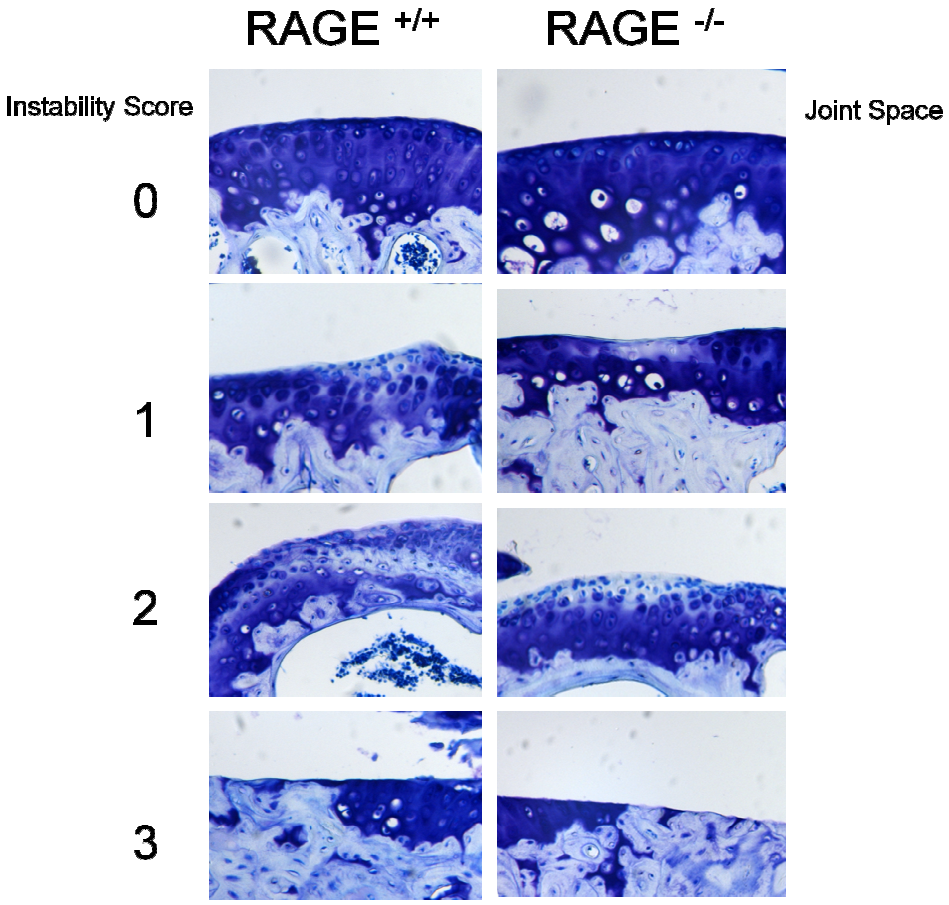


FIGURE 4.4
Proteoglycans loss was increased with increasing instability. Mouse knee sections were stained with Toluidine blue as described in the Methods. Loss of blue staining indicates loss of proteoglycans.

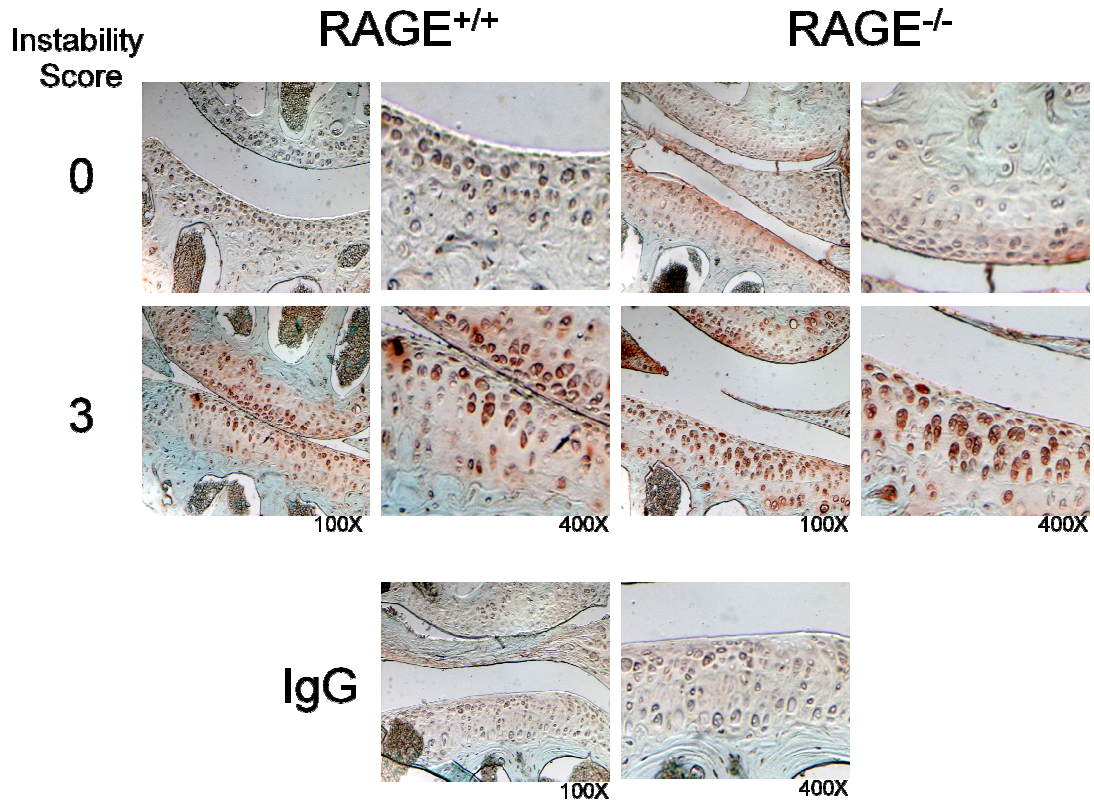


FIGURE 4.5
Appearance of type X collagen expression by surgical-induced instability is similar in RAGE$^{+/+}$ and RAGE$^{-/-}$ mouse knees. Mouse knee sections were analyzed by IHC for type X collagen two months after surgery. These data are representative of at least 3 different mouse donors and shown at 100X and 400X.

CHAPTER 5

Conclusion

5.1 SUMMARY

The studies in this dissertation examined the hypothesis that RAGE and RAGE ligands (specifically S100A11) contribute to the pathogenesis and progression of OA by promoting chondrocyte hypertrophic differentiation and concomitant cartilage degradation.

RAGE and several RAGE ligands are upregulated in aging and OA cartilage [146, 162, 163, 203]. Since increased RAGE expression is induced by an accumulation of ligands, it is not known if the increased expression of RAGE seen in OA is independent of the increased expression of ligands [94].

In these studies, the upregulated S100A11 expression in OA cartilage was localized to the pericellular matrix, suggesting that this calgranulin was actively secreted. Stimulation with pro-inflammatory cytokines IL-1β and TNFα and chemokine CXCL8 induced chondrocytes to release S100A11. Intracellular S100A11 mediates cytoskeletal rearrangements, cell growth and apoptosis [100, 134, 136, 139], but extracellular S100A11 exerts cytokine-like effects as evidenced by the induction of the pathologic hypertrophic chondrocyte phenotype.

S100A11-stimulated type X collagen expression was inhibited by sRAGE, a function-blocking antibody to RAGE, dominant negative RAGE and in $RAGE^{-/-}$ chondrocytes and cartilage explants. Though many RAGE ligands are recognized by other receptors and cell-surface structures [94, 144], these results reveal that S100A11 induced type X collagen expression and concomitant cell size increase in a RAGE-dependent manner.

Unexpectedly, CXCL8- and TNFα-induced type X collagen expression was also inhibited by sRAGE and RAGE-specific blocking antibodies. These results imply that the alteration in chondrocyte differentiation was not exerted directly through the CXCL8 receptor(s) (CXCR1/2) or the TNF receptor(s) (TNFRI/II), but rather by stimulating the release of RAGE ligands. As CXCL8 and TNFα stimulated the secretion of S100A11, it is probable these pro-inflammatory molecules stimulate the secretion of other RAGE ligands.

OA is not only characterized by chondrocyte differentiation to hypertrophy [23], but also by dysregulation in the balance between anabolism and catabolism [12]. In OA, the catabolic effects of cytokine-induced MMPs and aggrecanases (ADAMTS-4 and -5) soon overwhelm the attempt at matrix synthesis and repair [152, 155]. The preference toward catabolism stimulated by S100A11 was inhibited in $RAGE^{-/-}$ cartilage explants as measured by GAG release and the aggrecanase-induced neoepitope NITEGE generation on aggrecan. In addition, S100A11-inhibited PG synthesis was blocked in $RAGE^{-/-}$ chondrocytes (unpublished observation). Thus, S100A11 has the capacity to disrupt the equilibrium between the catabolic and anabolic pathways by a RAGE-dependent mechanism.

The immunoglobulin superfamily of receptors, including RAGE, are closely related to the cytokine receptor superfamily; dimerization and trimerization of the receptor is believed to enhance activation of intracellular signaling cascades [172-174]. Multimeric forms of certain S100/calgranulins appear to be associated with their extracellular activity by initiating an aggregation of RAGE, yet the conformation achieved by the type of bond formed is critical for propagation of signal [123-125].

In that context, S100A11 dimer formation and consequent function was examined. Oligomerization of S100A11 into an anti-parallel configuration is stimulated by TG2 transamidation at the N-terminal K3 in alpha helix I and the C-terminal Q102 domain in alpha helix IV [121]. Significantly, TG2 modulates CXCL8-induced chondrocyte hypertrophy and CXCL8 stimulates secretion of multimeric S100A11 in normal human chondrocytes [57, 162]. The multimers of S100A11 in extracts of human OA cartilage maintain multimerization under reducing conditions, suggesting the monomers were not linked together by noncovalent interactions or disulphide bonds.

In these studies, $TG2^{-/-}$ chondrocytes and cartilage explants were protected from S100A11-induced chondrocyte hypertrophy and GAG release. Though TG2 is a multifunctional enzyme with both ATPase/GTPase and crosslinking activity [167], it was demonstrated here that the catalytic transamidation activity was critical for S100A11 function in chondrocytes and cartilage explants. Specifically, the TG2 transamidation substrate site mutant, S100A11 K3R/Q102N (which does not multimerize either independently or when enzymatically-forced) failed to induce hypertrophy, the aggrecan neoepitope NITEGE or GAG release in cartilage explants. Thus, like other S100/calgranulins [178, 179], the style in which S100A11 is multimerized and its subsequent conformation appear essential for the pro-inflammatory effects in chondrocytes.

As a member of the MAPK family of signaling molecules, p38 has been implicated in the regulation of pathogenesis in rheumatoid arthritis by modulation of TNFα, CXCL8 and NF-κB [204]. However, p38 has been shown to also modulate differentiation, proliferation and maturation and is required for increased type X collagen

expression and decreased type II collagen expression in chondrocytes during endochondral ossification [205]. CXCL8 induces type X collagen expression in human articular chondrocytes by p38 MAPK activation and S100B modulates chondrocyte activation through regulation of NF-κB [57, 91].

In these studies, S100A11-induced type X collagen expression and GAG release was inhibited by a dominant negative form of the p38 upstream kinase, MKK3, in normal chondrocytes or in $MKK3^{-/-}$ cartilage. In addition, when RAGE had lost the capacity to transduce signals (dominant negative RAGE), p38 MAPK was no longer activated by S100A11. Furthermore, p38 MAPK was only activated by TG2-modified dimeric or multimeric S100A11. Thus, the activation of the MKK3-p38 MAPK pathway by S100A11 ligation of RAGE provides a critical link between differentiation and propagation of inflammation resulting in chondrocyte hypertrophy and disruption of the catabolic-anabolic balance.

The *in vitro* and *ex vivo* studies in this dissertation have implicated a role for RAGE and S100A11 in the pathogenesis of OA. Therefore, the functions of these molecules were examined *in vivo* on a surgically-induced instability model of OA. Though it has been criticized as an ineffective model, the ACL-T "stab" model has been well characterized in dogs and rats [73, 75, 78]. As spontaneous OA tends to develop in the medial side of the mouse knee, destabilizing the knee by ACL-T accelerates the natural medial compartment cartilage damage which results in progressive OA after 4 weeks [73]. As predicted by the model [73], the severity of OA in these studies directly correlated with the level of instability of the ACL with insignificant cartilage damage in the sham control animals, suggesting successful surgical technique. Nevertheless,

RAGE null joints were not protected from cartilage damage associated with OA as measured by total joint scores, PG content, osteophyte formation and type X collagen expression.

There are two reasons that could explain why RAGE null cartilage was not protected. First, OA induced in the ACL-T model was too severe to overcome the protective effects of one knocked-out protein, as suggested by the contradictory results observed in the IL-1β null mouse subjected to two different models of OA [44, 46]. Secondly, RAGE plays a less important role in modulating OA pathogenesis than its ligands, as demonstrated by the global protective effects of sRAGE in other disease models [97-99, 184]. RAGE is a pattern recognition receptor recognizing vastly different ligands including S100/calgranulins, HMGB-1 and Advanced Glycation Endproducts (AGEs) [83-86]. However, RAGE ligands are as promiscuous as their receptor and there have been questions raised as to the level of involvement of other receptors and cellular structures in mediating the effects of RAGE ligands [143].

In conclusion, there is an inflammation-induced accumulation of multimeric S100A11 in OA articular cartilage and concomitant increase in RAGE expression. TG2-modified S100A11 stimulates chondrocyte hypertrophic differentiation and dysregulated matrix catabolism by a RAGE and MKK3-p38-dependent mechanism. Thus, RAGE, S100A11 and TG2 share an important association to translate low-grade cartilage inflammation into the progression of OA. Yet, the role of RAGE and RAGE ligands in the pathogenesis of OA has been incompletely studied *in vivo* to definitively describe a pathogenic function. Therefore, a model of inflammation-mediated TG2, S100A11 and RAGE induction of chondrocyte differentiation and dysregulated matrix catabolism is

shown in Figure 5.1. It is important to note that S100A11 is not the only possible RAGE ligand in OA cartilage or that RAGE is not the only possible structure to recognize these ligands.

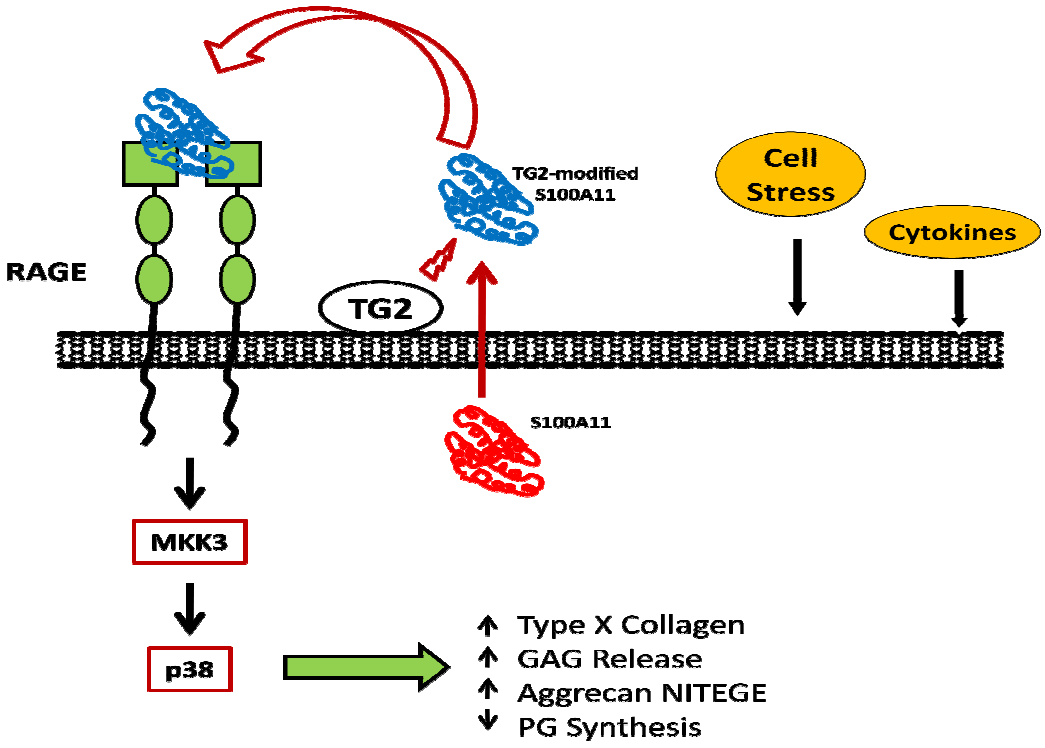


FIGURE 5.1
Proposed mechanistic model of the role of TG2-modified S100A11 and RAGE in stimulating chondrocyte hypertrophic differentiation and dysregulated matrix catabolism. Cell stress and inflammation can induce the release of S100A11 (and most likely other RAGE ligands) as well as promote the secretion and activation of TG2. Catalytically active TG2 transamidates extracellular S100A11. The multimeric conformation of the TG2-modified S100A11 can ligate and most probably cluster RAGE, signaling though the MKK3-p38 MAPK pathway to induce the chondrocyte hypertrophic differentiation and dysregulated matrix catabolism characteristic to OA.

5.2 PERSPCTIVES

OA is characterized by progressive cartilage degradation associated with chondrocyte differentiation and apoptosis, resulting in pain and impaired mobility [9]. OA is second only to ischemic heart disease as a cause of work disability in men over age 50 [206]. It is estimated that as of 2003, more than 26 million Americans have symptomatic OA and the prevalence of disease is expected to increase dramatically over the next 20 years as the population ages [206, 207].

Despite its public health impact, this disease continues to be a relatively unaddressed health issue and currently there are no approved disease modifying OA drugs (DMOADs). Traditionally, medical therapy of OA has been limited to lifestyle modifications, such as losing weight and exercise, and the treatment of symptoms, either locally or systemically [208].

As pain is the most common symptom, the non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most widely prescribed drugs. However, gastrointestinal intolerance, cardiovascular perturbations and renal toxicity have restricted their use in many patients [209]. Alternative and/or complementary therapies have included intra-articular injections of corticosteroids and synthetic hyaluronic-acid derivatives, which can reduce joint inflammation, treat pain and potentially restore mobility [210, 211]. Though these treatments focus on the non-specific relief of pain and have the potential to restore function, the effects are transient with no ability to reverse the cartilage degeneration [209, 212]. Thus, true DMOAD therapy must target the molecular pathways that modulate progressive joint destruction. Several potential therapeutic targets have been identified, and many compounds have been tested in preclinical and clinical trials [209].

Non-pharmaceutical, or nutraceutical agents, including glucosamine and chondroitin sulphate are capable of reducing radiographic knee OA progression. Chondroitin sulphate also works as an anti-inflammatory agent with a potential chondroprotective function by directly modulating the cartilage architecture [213, 214].

It is still unclear if subchondral bone sclerosis precedes OA or is a result of cartilage degeneration. Some studies suggest that factors regulating bone resorption are upregulated in OA [215, 216]. Bisphosphonates have the ability to suppress bone turnover and treatment with risedronate resulted in moderate improvement of OA symptoms, with significantly reduced markers of cartilage degradation and decreased subchondral bone lesions, but failed to reduce radiographic progression of knee OA compared to placebo [217]. Calcitonin can also affect bone remodeling, is an anabolic factor in cartilage and significantly reduced cartilage lesion size in experimental dog OA [218, 219]. In addition, modulating other important factors in bone resorption, including the receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG), and osteopontin (OPN), which also is modified by TG2, could have potential therapeutic effects [220-222].

Synovial inflammation and the concomitant release of inflammatory mediators contribute to the pathogenesis of OA [152]. The pro-catabolic role of IL-1β has been well established [159] and agents that regulate its synthesis or activity have been proposed and/or evaluated as an OA therapy. Clinical trials with diacerein, which inhibits IL-1β production from synovial tissue and cartilage, resulted in a modest improvement in radiographic hip OA, with limited reduction in pain but had no

significant cartilage-modifying effect in knee OA [223-225]. Blocking the activity of the IL-1ß-converting enzyme (ICE or caspase-1) inhibits the formation of active IL-1ß and IL-18. The ICE inhibitor, pralnacasan, reduced joint damage in two experimental models of murine OA [226]. Both *in vivo* and *ex vivo* treatment of OA cartilage with recombinant human IL-1 receptor antagonist (IL-1Ra) demonstrated a protective effect [227]. However, in a clinical trial, intra-articular IL-1Ra (Anakinra) injection for symptomatic knee OA showed no statistical improvement over placebo [228]. In addition, attempts at preventing IL-1β intracellular signaling have been considered and a specific extracellular signal-regulated protein kinase (ERK 1/2) inhibitor reduced cartilage lesions in an experimental rabbit model of OA [229].

Inhibiting other down-stream inflammation-induced mediators of cartilage destruction has been explored. Oxidative damage and chondrocyte apoptosis initiated by inducible nitric oxide synthase (iNOS) is associated with OA. iNOS deficient mice do not develop experimental OA and inhibition of iNOS reduced cartilage catabolism in an experimental canine model of OA [230-232].

Cartilage catabolism is regulated by both MMPs and aggrecanases, and the dysregulation of these enzymes results in the pathologic breakdown of the extracellular matrix [9, 183]. Since doxycycline can inhibit some MMPs [233], its effect was examined in the progression of human knee OA demonstrating some radiographic improvement compared with controls [234]. However, non-specific MMP inhibition has resulted in unfavorable musculoskeletal effects, thus targeted MMP inhibition (i.e. MMP-13, since it preferentially targets type II collagen) would be a more safe and effective treatment [235, 236].

The results presented in this dissertation provide evidence for RAGE and/or RAGE ligands as potential therapeutic targets for OA. Soluble RAGE (sRAGE) may contribute to the sequestration of various pro-inflammatory ligands and has been implicated in improved pathology in animal models of autoimmunity, diabetes and neurologic conditions [97-99, 184]. Decreased levels of circulating sRAGE have been demonstrated in the sera of patients with RAGE-associated disorders, including atherosclerosis, hypertension, rheumatoid arthritis, Alzheimer's disease, and diabetes [237-242]. However, to date, no studies have measured plasma or synovial fluid levels of sRAGE in OA patients. Furthermore, the studies in this dissertation did not monitor for constitutive or stimulated chondrocyte release of sRAGE.

Treatment could occur using a direct administration of recombinant human sRAGE to the problematic joint similar to corticosteroid or hyaluronic-acid therapy [210, 211]. Alternatively, activation of factors that increase endogenous expression of the soluble receptor could be employed. A recent study demonstrated that sRAGE can be cleaved from the membrane by ADAM10; however, this metalloproteinase has been implicated in acceleration of cartilage degeneration [81, 243]. Interestingly, preclinical studies have demonstrated that angiotensin-converting enzyme (ACE) inhibition stimulates the release of three distinct alternative splice variants of sRAGE in renal tissues of diabetic animals, and subsequently sequesters AGEs [244]. Therefore, it would be of interest to explore this possible affect in OA patients who are already prescribed ACE inhibitors.

Although several biochemical pathways have been identified for potential OA treatment, implementing the therapies still has many challenges. Reliable biomarkers

detected in serum or urine that reflects anabolic and catabolic activities of cartilage need to be established. Several candidates have been identified since they are elevated in OA: cartilage oligomeric matrix protein (COMP) and hyaluronic acid as markers of synovitis, C-Reactive protein (CRP) as a marker of systemic inflammation, and C-terminal cross linking telopeptides of collagen type II (CTXII) as a marker of cartilage degradation [245].

More sensitive imaging techniques need to be employed to monitor progression of cartilage degeneration or restoration of cartilage architecture. Traditional radiographic images cannot detect early alterations within cartilage and bone, and results could be interpreted differently depending on the position of the joint [246]. However, cartilage thickness, synovitis, bone marrow edema, and the changes in chondrocyte biochemical properties can be quantified by MRI [247-250]. Furthermore, quantitative articular surface curvature can be measured by MRI and could potentially identify different stages of OA [251].

Several of the potential DMOADs have been validated in large animal OA models [229, 231, 232]. However, it would be more financially efficient and effective to identify potential targets for DMOAD intervention using knockout mice. Nevertheless, there is controversy as to the surgically-induced model(s) that provide the most accurate representation of human OA, but with limited destabilization to not overwhelm the contributions of potential DMOAD targets [78].

In conclusion, although there are no approved DMOAD therapies, several biochemical pathways involved in the pathogenesis of OA have been identified, specific biomarkers have been discovered and more sensitive imaging techniques have been

developed. Therefore, in time, specific therapeutics for OA that modulate the cartilage, synovium and bone structure while relieving pain and improving function should be developed.

APPENDIX

Methods

Reagents

All-*trans* retinoic acid (ATRA) and human recombinant CXCL8 and TNFα were from R&D Systems. Soluble human RAGE (sRAGE), rabbit polyclonal RAGE specific functional blocking antibodies and human RAGE and dominant-negative (DN) RAGE cDNA in the vector pCDNA4-V5His (Invitrogen) were generously provided my Dr. AM Schmidt (Columbia, NY) DN-MAPK kinase 3 (MKK3) was generously provided by Dr. J. Han (The Scripps Research Institute, La Jolla, CA). Unless otherwise indicated, all other reagents were obtained from Sigma-Aldrich.

Mice

All mouse procedures were humanely performed and were reviewed and approved by the institutional animal care research committee. We established breeding colonies of RAGE null mice (from Dr. AM Schmidt, Columbia University, NY) and TG2 null mice (from Dr. Bob Graham, Victor Chang Research Institute, Darlinghurst, New South Wales, Australia), each on a C57BL/6 background and congenic wild type mice were used as controls**.** C57BL/6 wild type controls were purchased from The Jackson Laboratory.

Site directed mutagenesis and preparation of recombinant proteins

The QuickChange site-directed mutagenesis kit (Stratagene) was employed using normal human S100A11 cDNA to make the S100A11 K3R/Q102N mutant or normal human TG2 cDNA to make the TG2 GTP binding site mutant K173L and the TG2

transamidation active site mutant C277G [119]. K3R forward: 5' GCTCCAACATGGCAAGAATCTCCAAGC 3', K3R reverse: 5'-CTTAGGGCTGGAG ATTCTTGCCATG-3', Q102N forward: 5'CAAGGCTGTCCCTTCCAACAAGCGGA-3' and Q102N reverse: 5'-CCTCAGGTCCGCTTGTTGGAAGGGAC-3'.

Recombinant forms of His-tagged TG2 and S100A11 K3R/Q102N were purified from transfected human fibroblastic HEK-293 cells using the Probond Purification Kit (Invitrogen). After purification through binding to nickel columns, dialysis and concentration, the preparation was tested with Limulus Amebocyte Lysate QCL 1000 assay (Cambrex) and determined to have <0.1EU/ml (or below the detection limits) of endotoxin.

For $CuCl_2$ oxidation of S100A11 to generate S-S bonded oligomers [169], 1 μg rhS100A11K3R/Q102N was incubated in 6 μM $CuCl_2$ in 20mM Tris-HCl for 1 hour, after which 90 μM copper chelating diethylenetriamine-*N,N,N',N'',N''*-pentaacetic acid was added and the reaction products studied by SDS/PAGE under non-reducing or reducing conditions.

Studies of mouse femoral head cartilage explants

We adapted the methods of Glasson *et. al.* [155] to study femoral head cartilage caps from mice 2 months of age. In brief, isolated femoral head caps were placed in a 96-well plate containing 0.05 ml DMEM/high glucose supplemented with 10% FCS, 1% L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO_2 for 18 hours prior to the described treatments.

Glycosaminoglycans (GAG) release into conditioned medium at 48 hours was measured spectrophotometrically in triplicate at 525 nm using 200X 1,9-Dimethyl-Methylene Blue (DMB) in wells containing 5 μl media and 100 μl 0.4 M Glycine, 0.4 M NaCl, 0.095 M HCl, pH 3.0, with chondrotin sulfate used as the standard [252]. Nitrite level to assess NO generation was assayed by the Griess reaction [57]. We assayed TG transamidation catalytic activity by 5-biotinamidopentylamine binding to dimethylcasein [57], with 1 Unit of TG designated as 1 μM substrate catalyzed per hour.

Immunohistochemistry and Immunocytochemistry

9 μm frozen sections were fixed using 100% Ethanol for 10 min and allowed to dry for 30 min. Sections were then incubated with 1% Triton X-100 for 5 min or with 0.1 U Chondroitinase and 0.1 U Keratanase for 1 hour at 37°C, washed 2 times with PBS containing 0.05% Tween-20 (T-PBS) and blocked with 1% BSA/1% casein for 30 min at 37^{o}C, then incubated for 1 hour at 37^{o}C with primary antibody in the same blocking buffer. After three washes, primary antibody was detected via the avidin-biotin conjugate method using the Histostain-Plus reagent (Invitrogen) and with Fast Green (0.001%) added for 5 min followed by two washes in water. Whole serum containing rabbit polyclonal antibodies to type X collagen used for immunohistochemistry were from Cosmobio at a 1:100 dilution. Rabbit polyclonal antibodies to the aggrecanase neoepitope NITEGE were from GeneTex and used at 10μg/ml.

For immunocytochemical analysis, chondrocytes were plated on glass cover slips and after 3 days the cells were fixed in 4% paraformaldehyde, and then blocked with

1%BSA/1% casein for 1 hour. Primary antibodies (diluted to 10 μg/ml) were added for 1 hour, and after 3 washes the primary antibody was detected via the avidin-biotin conjugate method using the Histostain-Plus reagent (Invitrogen) applied according to manufacturer instructions. All light microscopy images were visualized on a Nikon microscope using the 4X and 10X objective lenses and with 10X binoculars, and Nikon digital camera images were captured using ACT-2U software.

Defined specimens of normal and OA human articular cartilage (age ranges, 22–32 and 66–83, respectively) were taken as full-thickness blocks. For immunohistologic analyses of RAGE and S100A11, frozen sections (5 μm) were fixed using 4% paraformaldehyde for 20 min. After washing with PBS, the sections were incubated with 1% Triton X-100 and microwaved for 1 min, blocked with 10% goat serum for 20 min, and incubated for 24 h at 4^{o}C with mouse anti-RAGE or anti-S100A11 Abs. The primary Ab was detected via the avidin-biotin conjugate method applied according to manufacturer's instructions using the Sigma Rabbit ExtrAvidin Peroxidase Staining kit. Peroxidase activity was detected using the Sigma Fast DAB staining kit, according to manufacturer's instructions.

Murine chondrocyte isolation

To isolate immature mouse knee articular chondrocytes, patellar groove and femoral condyle cartilage dissected out from 7-8 day old mice were digested using 3 mg/ml Collagenase D (Roche) in DMEM High Glucose containing 10% serum for 45 min, then transferred into 2 mg/ml Type II collagenase for an additional digestion of 3 hours. The supernatant was filtered through a 70 μm cell strainer and the cells were

centrifuged at 500 x *g* for 5 min, washed in PBS and plated in monolayer culture in DMEM/high glucose supplemented with 10% FCS, 1% L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO_2. All experiments were performed after 3 days. Chondrocyte differentiation was validated via RT-PCR for type II collagen (forward: 5'-GCCCGTCAGGAAGTACC-3' and reverse: 5'-ACCAGCATCTCCTTTCTGT-3') and aggrecan (forward: 5' TTCCATCTGGAGGAGA GGG 3' and reverse: 5' ATCTACTCCTGAAGCAGATGTC 3', using type I collagen (forward: 5'-CCCTGGTATGACTGGCTT-3' and reverse: 5'-GACCACGAATCCCTTC CT-3') as a negative control.

Studies of cultured mouse chondrocytes, primary human chondrocytes and human chondrocytic CH-8 cells

Murine chondrocytes were carried in monolayer culture in DMEM/high glucose supplemented with 10% FCS, 1% L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin at 37°C with 5% CO_2. Where first passage chondrocytes were stimulated with agonists such as S100A11, serum supplementation in the medium was decreased from 10% to 1%.

Primary human chondrocytes were obtained from the medial and lateral condyles, the patellar groove and the tibial plateau of normal human knee articular cartilages (donor age range, 22–62) [57]. Transient transfection of human articular chondrocytes was performed using a Nucleofector apparatus (AMAXA) and modified by substitution of the transfection reagent with Fugene 6 (Roche), with achievement of ~70% transfection efficiency in human chondrocytes.

CH-8 cells, an SV40 immortalized clone of normal human knee articular chondrocytes [253], were a generous gift of Dr. M. Hiramoto (Nihon University School of Medicine, Tokyo, Japan). These cells were studied between passages 5 and 15 under the same culture conditions described above, under which maintenance of type II collagen and aggrecan expression were confirmed by RT-PCR. Transient transfection of CH-8 cells was performed using Lipofectamine plus (Invitrogen) according to manufacturer instructions, with ~60% transfection efficiency.

Flow cytometric analyses

Human articular chondrocytes (1X 10^6) were permeabilized using the BD Cytofix /Cytoperm kit (BD Pharmingen). Where indicated, the cells were incubated for 60 min at 4°C with murine monoclonal anti-RAGE or murine IgG isotype control. Washed cells were incubated with FITC-conjugated goat F(ab')2 anti-mouse IgG for 60 min at 4°C. Fluorescence was detected using a FACSCalibur apparatus (BD Biosciences) with data analyzed using CellQuest software (Purdue University, West Lafayette, IN).

shRNA design and characterization

Ambion's web-based shRNA design program was used to identify 21-mer regions within TG2 effective for shRNA targeting. Five sequences were originally tested to find an optimal sequence. The 21-mers were then used to generate the 55bp oligos which included two 19bp regions specific to human TG2 complementary to each other to form the hairpin, a loop sequence separating the complementary domains and a dinucleotide overhang that can hybridize with the RNA target (part of the original 21-mer). The two

55bp complementary oligos were annealed and then ligated into the pSilencer 4.1-CMV neo vector (Ambion). The scrambled TG2 shRNAs were randomly generated with the same basepairs as the siTG2. After sequence confirmation, the vectors were transfected into human articular chondrocytes, using the AMAXA as described. The optimal 19 bp sequences for human TG2 is 5'-GAGCGAGAT GATCTGGAAC-3'(1116-1132)

SDS-PAGE/Western blotting

For SDS-PAGE / Western blotting analyses, cell lysates were harvested in 10mM Tris (pH 7.6), 150mM NaCl, 0.5mM EDTA, 1mM EGTA, 1% SDS containing a freshly added Complete Protease Inhibitor Cocktail tablet (Roche). Aliquots of 0.01-0.05 mg protein from each sample were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose [130].

Polyclonal antibodies in rabbit serum diluted 1:3000 for type X collagen were from Calbiochem. Rabbit polyclonal antibodies recognizing the human S100A11-specific peptide CHDSFLKAVPSQKRT (diluted to 1 μg/ml) were generated by Zymed Laboratories. Rabbit polyclonal antibodies to phosphorylated p38 and total p38 were from Biosource (diluted to 1 μg/ml). Polyclonal antibodies specific for TG2 in goat serum were diluted 1:2000 (Upstate). HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG employed at 0.1 μg/ml were obtained from Pierce and immunoreactive products were detected using enhanced chemiluminescence (ECL).

Assays of S100A11 binding to cells

For assays of binding of S100A11 to cells, aliquots of 1 X 10^3 CH-8 cells were plated in a 96-well plate for 18 hours, after which the cells were incubated with 20 μg/ml goat anti-RAGE antibodies or normal goat IgG for 30 min at 4°C. After 2 washes in growth medium, 1 μg/ml S100A11 or K3R/Q102N S100A11 was added for 1 hour at 4°C.

The polyclonal anti-S100A11 antibodies were added at 1 μg/ml for 1 hour at 22°C after 3 washes, and after 3 further washes, biotin-labeled anti-rabbit IgG was added at 1 μg/ml for 1 hour at 22°C. After 3 subsequent washes, Steptavidin-alkaline phosphatase (Invitrogen) was added for 1 hour at 22°C, and after 3 further washes, binding of S100A11 was detected using the Alkaline Phosphatase Yellow (pNPP) Liquid Substrate (Sigma). Cell binding of S100A11 was normalized to protein concentration per sample.

ACL-T "stab" OA model

Mice were anesthetized and the surgical site is cleaned with alcohol. The knee was bent and the skin was pulled over the patella. A 28.5 gauge needle was inserted through the patella into the groove between the femoral condyles. The needle was "stabbed" three times, going in only past the bevel so as to prevent rupture of the popliteal artery. The success of ACL transection was immediately checked by presence of the anterior drawer sign (free lateral movement of the tibia in respect to the femur) [73]. We have verified that this procedure, in our hands, does not significantly damage knee ligaments other than the ACL.

Histopathology of OA model

Eight weeks after surgery, the mice were sacrificed and sent for evaluation to Alison Bendele, DVM, PhD at Bolder Biopath. Preserved and decalcified mouse knees were processed through graded alcohols and a clearing agent, infiltrated and embedded (in the frontal plane) in paraffin, sectioned, and stained with toluidine blue. Joints that had histological evidence of instability induction were identified by the presence of proliferative changes in the medial synovium, marginal zones, collateral and cruciate ligaments. An instability score was recorded for each joint according to the following criteria: 0=No instability; 1=Minimal to Mild instability (Minimal to mild proliferative changes in ligaments, synovium and marginal zones); 2=Moderate instability (moderate proliferative changes in ligaments, synovium and marginal zones); 3=Severe instability (severe proliferative changes in ligaments, synovium and marginal zones).

The medial and lateral femoral and tibial cartilage degeneration were scored for severity of the cartilage assigned with attention to zonal (inside, middle, and outside) distribution of lesions using the following system: 1=superficial damage, tangential layer of collagen absent over 50% or greater of the zone surface or up to 10% loss of PG and/or chondrocytes in focal or diffuse distribution in zone. 2=matrix loss extends into upper ¼ of 50% or greater area of the zone or up to 25% loss of PG and/or chondrocytes in focal or diffuse distribution in zone. 3=matrix loss extends through ½ of the cartilage thickness over 50% or greater of the zone or there are focal areas of full thickness loss that are up to 25% of the width of the zone or up to 50% loss of PG and/or chondrocytes in focal or diffuse distribution in zone. 4=matrix loss extends through ¾ of cartilage thickness over 50% or greater of the zone or there are focal areas of full thickness loss

that are 26-50% of the width of the zone or up to 75% loss of PG and/or chondrocytes in focal or diffuse distribution in zone. 5=matrix loss extends through entire cartilage thickness over 50% or greater of the zone or up to 100% loss of PG and/or chondrocytes in focal or diffuse distribution in zone. The scores for each third were summed for each area of the joint and then the whole joint. Total joint score has a maximum of 60.

Scoring of osteophytes was performed with an ocular micrometer. Small osteophytes measured up to 150μm, medium osteophytes measured 151-300μm, and large osteophytes measured >301μm.

Statistical analyses

Where indicated, error bars represent SD. Statistical analyses were performed using the Student's *t* test (paired two-sample testing for means).

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