The Effects of Non-Surgical Interventions on Osteoarthritis-Like Changes in the Mouse Knee

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy School of Aging Studies College of Arts and Sciences University of South Florida

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Dedication

I dedicate this to my daughter, Aviendha, who has been right there every step of the way. You have experienced and endured more than most 10 year olds in this process. My hope is that it inspires (not disheartens) you to continually ask questions and seek answers. You are a truly special person and a great gift from God. Thanks for your patience, flexibility, encouragement, fun-loving personality, smiles, and hugs. I couldn’t ask for a better daughter. I hope that this research leads to more research and many more answers, so that one day your generation will not experience the debilitation that can come with osteoarthritis.

And, above all else, I dedicate this to God who provides strength beyond measure and encouragement at just the right time. I have clung to Joshua 1:9 over the past years. Every day it is more apparent to me that we truly are fearfully and wonderfully made. What a great blessing it is to “discover” parts of Your marvelous creation. The more I learn, the more I am humbled by Your greatness.

Wendy
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The Effects of Non-Surgical Interventions on Osteoarthritis-Like Changes in the Mouse Knee

Wendy K. Anemaet

ABSTRACT

Osteoarthritis (OA) is a debilitating condition affecting over 21 million persons in the United States. This number is expected to rise in the coming decades. Treatment approaches for OA focus on symptom modifying measures (i.e., pain relief) as disease modifying interventions do not currently exist. However, some of the interventions used to alleviate the symptoms of OA are also thought to have disease-modifying benefits. Two such non-surgical interventions for OA are intra-articular hyaluronan (HA) injections and physical exercise. In order to effectively study their effects in human OA, animal models that are amenable for studying intervention outcomes are needed.

The research focused on developing and characterizing a progressive non-surgical model of knee OA in adult mice. This model was used to firstly, examine the capacity of intra-articular HA injections to prevent knee joint degeneration, and secondly to examine the capacity of moderate exercise to prevent onset and progression of joint degeneration.
Intra-articular injections of TGF-β1 into murine knees produce synovial hyperplasia, osteophyte formation, and fibrotic changes on cartilage surfaces and joint capsules. However, additional exposure of the joints to high intensity treadmill running (biomechanical overuse) results in more widespread and focal OA-like cartilage erosions of both the tibial and femoral surfaces, similar to that described for the pathological appearance of late human knee OA. Taken together, these data support that synovitis and soft-tissue activation in early OA joints may precede and/or accelerate the process cartilage degeneration characteristic of progressive and late stage osteoarthritis.

Intra-articular injections of high molecular weight HA one day following TGF-β1 injections resulted in decreased synovial hyperplasia, minimized osteophyte formation, and significantly decreased severity of cartilage lesions. A four week, alternate day, low intensity aerobic treadmill running program prior to TGF-β injections and overuse also resulted in decreased severity of cartilage lesions.
Overview of Osteoarthritis

Definition

Osteoarthritis (OA), also known as degenerative joint disease (DJD), is a musculoskeletal disease that is diagnosed both structurally and clinically. Structurally, synovitis (Benito et al., 2005), progressive articular cartilage loss (Cahue et al., 2004; Cerejo et al., 2002), osteophyte formation (Boegard et al., 1998), and subchondral sclerosis (Yamada et al., 2002), generally in weight bearing joints such as knee and hip, give rise to biomechanically unstable joints that result in loss of function (Sharma et al., 2001). The resulting abnormal biomechanical forces on the joint tissues play a role in the erosion of cartilage surfaces as well as subchondral sclerosis.

Clinically, patients with OA experience pain, stiffness, loss of motion, weakness, and joint instability, all leading to functional limitation and disability (Altman et al., 1986). However to date, criteria to precisely stage the severity of the disease for treatment options remain ill defined (Boegard et al., 1998; Creamer et al., 1999; Felson et al., 2000a; McAlindon et al., 1992; O’Reilly et al., 1998; Peterson et al., 1996; Weidow et al., 2006). For example, persons may report a high level of pain with only mild radiographic changes, or conversely, may have dramatic radiographic changes with minimal reports of pain.
Development and application of assays of OA-specific biomarkers for synovial fluid, serum and urine have been the focus of active clinical and basic science research efforts, but have not yet resulted in clear marker panels that could be readily used in diagnosis and prognosis of OA in the clinic or in translational research studies (Bauer et al., 2006; Giles et al., 2007; Girling et al., 2006; Kloppenburg et al., 2007; Krause et al., 2006; Lohmander and Eyre, 2005; Nemirovskiy et al., 2007; Sumer et al., 2006; Thonar et al., 1993).

Incidence and Prevalence

Clinical diagnosis of OA is made in one in three persons over the age of 50 years, and women are more frequently affected than men (Lawrence et al., 1998). OA is the leading cause of chronic disability in the elderly (Felson et al., 2000), owing in part to improved longevity. One in two persons over the age of 70 years and over 85% of persons aged 80 years or more have a clinical diagnosis of OA (National Centers for Health Statistics, 2004). The most commonly affected joint is the knee, but OA also affects the hip, spine, hands, and feet (Glass, 2006). Incidence and prevalence vary slightly by the affected area. Women have higher incidence and prevalence of hand and knee OA and men have higher prevalence of hip OA (Jordan, 1996).
Etiology of Osteoarthritis

Genetics

Genetic defects of connective tissue structural and regulatory proteins, such as bone morphogenetic protein 2 (BMP2), cartilage intermediate layer protein (CILP), frizzled related protein (FRZB), and TNF alpha-induced protein (TNFAIP6) have been clearly implicated in the development of OA and are often associated with increased risk of OA (Lane et al., 2006; Valdes et al., 2004). For example, mutations in humans that increase susceptibility to OA involve collagens II (Vikkula et al, 1994), IX (Olsen, 1997), and XI (Jacenko and Olsen, 1995) genes and are often manifested as multiple joint involvement (Abel et al., 2006; Bateman, 2005; Clements et al., 2006; Hakim and Sahota, 2006; Lopponen, et al., 2004; Roughley et al., 2006; Williams and Jimenez, 2003; Zhang and Doherty, 2005). However, while genetic linkages to OA exist, the disease is rarely caused by a single genetic defect (Zhang et al., 1998), but instead can be considered a multi-genetic, multi-factorial class of diseases. (See Figure 1).

Injury

About 10% of all OA cases are post-traumatic in origin (Marsh, 2004), including articular fractures (Furman et al., 2006), work or sports related overuse of joints (Hansen and Reed, 2006; Hunt, 2006; McMillan and Nichols, 2005), and injuries to the anterior cruciate ligament and/or menisci (Bartz and Laudicina, 2005; Koh and Dietz, 2005; Roos, 2005).
The precise etiology of post-traumatic OA remains to be determined as individuals with no joint trauma and little repetitive activity also develop OA (Hannan et al., 1993; Schrier, 2004). Moreover, exercising or involvement in sports in the absence of muscular imbalances or physical damage to joint tissues does not result in increased OA (Cymet and Sinkov, 2006; Schrier, 2004). Thus, it is more likely that the extensive remodeling of joint tissues (synovium, cartilage, bone, ligament, meniscus) which occurs in response to injury generates the metabolic disturbance in the whole joint that eventually leads to the pathological manifestation of OA (Felson, 2004).

Obesity

Obesity has been found to be strongly linked to knee OA (Cooper et al., 2000; Schouten et al., 1992). Persons who were overweight at age 37 (when knee OA is uncommon) had an increased risk of developing knee OA at age 70 or older (Felson et al., 1988). In women between 20-89 years with a body mass index (BMI) of > 25 kg/m², a new diagnosis for symptomatic knee OA was four-to ninefold higher than for age-matched controls with BMI < 25 kg/m² (Felson, 1990). In addition, obesity is linked to enhanced progression of OA as defined by radiographic changes (Schouten et al., 1992; Spector et al., 1994) and may result in increased joint pain and disability (Jinks et al., 2006; Mallen et al., 2007; Verbrugge et al., 1991).

It has been suggested that accumulation of adipose tissue results in altered levels of hormones, cytokines, and growth factors, increased bone
density, and changes in immune response (such as prolonged low grade inflammatory response), and all of the above may have a role in OA development (Nevitt and Felson, 1996; Olson et al., 2007). In addition, weight gain is common in persons with decreased functional status and/or disability, all hallmarks of OA and aging, potentially enhancing disease progression further in such individuals (Lievense et al., 2002).

Aging

Both the incidence (number of new cases in a specific period of time) and prevalence (the number of cases in a population at a given time) of OA increase in the elderly (Kopec et al., 2007). A common initiating factor in OA is the inability of the joint to repair mechanical (trauma) or metabolic (obesity) injuries (Olson and Marsch, 2004) during which cellular and biochemical pathways, including immune responses, are activated for tissue repair (Polyzois et al., 2006). Immunosenescence, therefore, may be an important factor in promoting pathogenesis of OA in the elderly. Age-related changes in the innate and acquired immune pathways manifest themselves in chronic inflammation and inefficiency in immune surveillance (Senchina and Kohut, 2007), including age-related changes in function of neutrophils and macrophages (Fulop et al., 1997; Gomez et al., 2005: Lord et al., 2001; Plackett et al., 2004; Stout and Suttles, 2005; Wenisch et al., 2000). As a result, older adults are more vulnerable to infection (Crichton and Puppione, 2006), and wound healing is slow or even impaired. Moreover, circulating levels of pro-inflammatory mediators including
interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α), prostaglandin, and C-reactive protein (CRP) can occur in the elderly (Franceschi et al., 2000).

Pathogenesis of Osteoarthritis

The pathogenesis of OA involves interactions of joint tissues, joint biomechanics, and biochemical pathways. The predominant pathological features of OA are synovitis and fibrosis, accelerated bone remodeling with osteophyte development, and articular cartilage degeneration. As outlined above, underlying characteristics, such as age, genetic background, and comorbidities (obesity) predispose an individual to the development of OA. In conjunction with aberrant joint biomechanics in the form of injury, overload, or joint instability, this predisposition may lead to altered biochemical pathways including those involved in cytokine and growth factor signaling, or matrix biosynthesis and turnover. These altered pathways lead to the development and progression of tissue damage in OA which present clinically as radiographic changes in bone and cartilage and joint pain. The presentation of pain is also affected by psychosocial and socioeconomic factors (Thumboo et al., 2002) as well as the presence of co-morbidities (Tuominen et al., 2007). The presence of pain, in turn, may act to alter biochemical pathways leading to further development and progression of OA. In addition, pain leads to disability and distress which lead to increased pain perception (Figure 1).
Figure 1: Representation of complex relationship between environmental and endogenous risk factors for joint damage, osteoarthritis, and joint pain and their consequences

(From: Dieppe and Lohmander, 2005)
The Human Knee Joint

The mammalian knee joint is comprised of two long bones, the tibia and the femur, and a sesamoid bone, the patella (Figure 2). The patella serves as a pulley allowing the quadriceps muscle to act more efficiently, sliding in the femoral sulcus.

Three major muscle groups control movement at the knee. The quadriceps is comprised of four muscles (the vastus lateralis, the vastus intermedius, the rectus femoris, and the vastus medialis). This muscle group is responsible for knee extension and is contiguous with the extensor mechanism (comprised of the patella tendon, patella, and patella ligament). Muscle imbalances, especially weakness of the vastus medialis, results in abnormal tracking of the patella. Abnormal pulling of the quadriceps muscle (from muscle imbalance or structural abnormalities) in other joint tissues results in the patella moving out of the femoral sulcus. The distal end of the femur, proximal end of the tibia, and posterior aspect of the patella are lined with articular cartilage. When the patella tracks abnormally, damage to the articular cartilage on the femur and patella may occur (Cahue et al., 2004; Sharma et al., 2001; Sharma et al., 2003).

The hamstrings consist of the semimembranosus, the semitendinosus, and the biceps femoris and all three are responsible for knee flexion. In addition, the hamstrings protect the integrity of the anterior cruciate ligament by counteracting shear through controlling anterior movement of the tibia on the femur. The third muscle acting at the knee is the gastrocnemius which is primarily responsible for ankle plantarflexion, but also assists with knee flexion.
Figure 2: Schematic of the knee joint (patella reflected)

(Diagram by Arnheim and Prentice accessed at http://factotem.org/library)
Ligaments about the joint provide stability. The patella ligament connects the patella and quadriceps muscle to the tibia at the tibial tuberosity and provides anterior-posterior stability to the joint. The anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL) cross at the center of the joint (Figure 2) connecting the tibia to the femur. They are intra-articular ligaments because they are within the joint capsule and surrounded by synovial fluid. They protect the knee against twisting and control forward sliding of the tibia on the femur (shear). The medial collateral (MCL) and lateral collateral (LCL) ligaments are located on the medial and lateral sides of the knee, respectively (Figure 2). They connect the femur to the tibia on the medial side and fibula on the lateral side. The MCL protects the knee against valgus (inward) forces and the LCL protects against varus (outward) forces.

Menisci are two crescent-shaped pieces of fibrocartilage between the femoral and tibial surfaces (Figure 2). They cushion the joint from impact and distribute compressive and shear forces across the articular cartilage surfaces.

Biomechanically the knee is at risk for injury as a result of the high torque moments created by the long bones and the frequency of use of the lower extremities. Dynamic loading of the joint (walking, running, and jumping) can increase joint forces up to 20 times relative to just standing. In addition, rapid changes of motion that occur with many activities as well as balance corrections require quick changes in velocity and impose additional stresses on supporting structures of the knee.
The Synovium

The synovial membrane is the delicate, vascularized tissue that covers the non-articular surfaces of the synovial joint cavity, including intra-articular ligaments (the anterior and posterior cruciate and patellar ligaments), the patellar tendon, and the intra-capsular bone surfaces (Figure 3). It is composed of an epithelial-like cell lining facing the joint cavity (intima), and a deeper, vascularized fibrous layer (subintima) that can also contain groups of differentiated fat cells. Synovial functions include innate immune surveillance, lubrication, and intra-articular joint tissue nutrition, representing a metabolic protection of joint function (Berumen-Nafarrate et al., 2002).

The synoviocytes in the lining have been defined ultra-structurally as Type A (macrophage-like) cells and Type B (fibroblast-like) cells (Pavolich and Lubowitz, 2008). Type A cells function as resident tissue macrophages to remove cell debris and products of macromolecular turnover processes which include synovial fluid hyaluronan (HA; Bondeson et al., 2006) lubricin, collagen, and aggregcan fragments. Type B cells are the major producers of HA and lubricin for the synovial fluid that decrease the joint friction coefficient reducing forces on the articular cartilage (Schmidt et al., 2007; Smith et al., 2003).

Alteration in cell and matrix constituents of the synovium, which is also known as synovitis, has been reported to correlate with progression of OA. Thus the pathological synovium may to a large extent be responsible for production of pro-inflammatory cytokines (interleukins, TNF and oncostatin M) and growth factors (platelet derived growth factor [PDGF], transforming growth factor-beta
[TGF-β1]). (Bondeson et al., 2006; Sutton et al., 2007) These in turn may regulate the expression of other pro-inflammatory pathways leading to the production of tissue destructive proteases including collagenases and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteases (Fukui et al., 2005; Youn et al., 2002).
Figure 3: Location of the synovium and synovial fluid within the knee joint

From http://www.healthcare.utah.edu/healthinfo/images
Bone

*Bone matrix* is primarily composed of *calcium hydroxylapatite* deposited within a collagen matrix. The mineral provides compressive strength and the collagen provides elasticity. The outer layer of *cortical* bone accounts for 80% of the total bone mass and a cell-rich porous network of rod- and plate-like *trabecular bone* accounts for the remaining 20% of the total bone mass (Figure 4). Trabecular bone, however, has about ten times the surface area of cortical bone and its porous network harbors blood vessels and marrow. The *periosteum* is a thin layer of connective tissue on the outer surface of bones. It has nociceptive nerve endings, provides the blood supply to the cortical bone, and contains progenitor cells that are activated during fracture healing (Fiedler et al., 2002).

Bone contains two major cell types (Figure 5): *osteoblasts* are bone-forming cells that make osteoid, a protein mixture of type I collagen, proteoglycans, and glycoproteins which mineralizes to become bone; and, *osteoclasts* are large multi-nucleated cell on the bone surfaces responsible for bone resorption (You et al., 2008).
Figure 4: Structure of bone

- Lacunae containing osteocytes
- Lamellae
- Canaliculi
- Osteon
- Periosteum
- Osteon of compact bone
- Trabeculae of spongy bone
- Haversian canal
- Volkmann's canal
Figure 5: Bone cell associations

Bones function to protect internal organs, build the skeleton to support the body, and produce movement in conjunction with muscles. In addition bones act as storage sites for minerals, especially calcium and phosphorus, and contain the hemapoietic (blood) cell development within the marrow. Bone can also buffer the blood against pH changes by absorbing or releasing alkaline salts and can chelate heavy metals thereby removing them from the blood and minimizing their toxicity on other organs of the body (Mutlu et al., 2007).

Several investigators have proposed that alteration in bone metabolism may play a role in the initiation and progression of OA (Botter et al., 2007; Muraoka et al., 2007; Radin, 1999; Sniekers et al., 2008). For example, recent studies looking at collagen synthesis and degradation reported an imbalance in those processes that may cause some of the OA-associated subchondral bone changes including subchondral sclerosis (Bailey et al., 2004; Day et al., 2004).

Osteophyte formation at joint margins in OA joints (Boegard et al., 1998) are another indication of bone adaptation associated with OA. Osteophytes develop in joints constrained by a joint capsule lined with synovium (van der Kraan and van den Berg, 2007). Mesenchymal progenitor cells within the periosteum (Figure 4) and the synovial lining (Figure 3) are involved in the development of osteophytes (Shirasawa et al., 2006). Their proliferation and migration followed by chondrogenic differentiation and cartilaginous matrix accumulation at the epiphyseal margins of bone and cartilage have been well described (van der Kraan and van den Berg, 2007). (See Figure 5). Subsequent
chondrocyte hypertrophy and endochondral ossification forms the mature osteophyte. TGF-β1 and insulin-like growth factor (IGF)-1 are both involved in the formation of osteophytes (van der Kraan and van den Berg, 2007; Okazaki et al., 1999), as are growth factors such as BMP-2 and BMP-4, and these are also typically products of macrophages (Blom et al., 2004; van Lent et al., 2004).

Articular Cartilage

Articular cartilage is a specialized connective tissue composed of an abundant extracellular matrix (ECM) and metabolically active cells, the chondrocytes. The chondrocytes in adult articular cartilage are responsible for producing and organizing the extracellular matrix in response to the normal physical-chemical demands generated by usage of the tissue during joint movement (Iannone and Lapadula, 2003). The extracellular matrix of cartilage is composed primarily of collagens II, IX, and XI and the proteoglycan aggrecan. In turn, by forming supramolecular complexes (collagen fibrils and proteoglycan/hyaluronan aggregulates) they provide the tissue with its viscoelastic properties to resist compression and dissipate sheer forces imposed on cartilage surfaces during articular joint usage.

Four distinct, histological zones have been identified in human cartilages (Figure 6). The superficial tangential zone is the outer layer facing the synovial cavity. It has the highest collagen content (85% by dry weight) and comprises 10-20% of the cartilage thickness. Chondrocytes in this superficial zone are
elongated and collagen fibrils are oriented parallel to the joint surface which may help to resist shear forces (Carter and Wong, 2003).

The transitional zone makes up about 60% of the cartilage thickness and contains less collagen (68% by dry weight) but abundant proteoglycan compared to the superficial tangential zone. Collagen fibers are larger in diameter and are more randomly oriented. Residual chondrocytes have a round morphology.

The deep zone comprises 30% of the cartilage thickness and has collagen fibers oriented perpendicular to the subchondral bone. Chondrocytes are round and arranged in columns. A calcified cartilage zone is at the interface with the bone, with few chondrocytes. This region also contains the calcified “tidemark” region that functions as a barrier to vascular penetration from the subchondral bone region (Langworthy et al., 2004).

Substantial efforts over the past several decades were directed towards understanding the molecular and cellular mechanisms by which the structural components of articular cartilage are irreversibly destroyed and result in progression of OA. Briefly, collagen fibrils are degraded by matrix metalloproteinase collagenases (MMPs) (Davoli et al., 2001; Pratta et al., 2003; Smith, 2006; Tardif, et al., 2004), whereas aggrecan molecules are substrates for ADAMTS proteases (Gao et al., 2004; Nagase and Kashiwagi, 2003; Sandy, 2006; Sandy and Verscharen, 2001; Song et al., 2007). Despite the extensive work on the protease identification, little is known about the temporal and spatial regulation of their activities in normal tissue turnover and OA (Cawston and Wilson, 2006; Sandy, 2006).
ADAMTS metalloproteases cleave aggrecan, a major component of cartilage extracellular matrix in human OA. It remains open which aggrecanase is responsible for aggrecan destruction during articular cartilage degeneration in human OA (Song et al., 2007; Tortorella and Malfait, 2008). However, the generation and use of ADAMTS-5 knockout (KO) mice demonstrate that activity of this enzyme is required for progression of OA-like cartilage lesions in mouse knees surgically destabilized by MCL transection (Glasson et al., 2005) or challenged by antigen-induced arthritis (Stanton et al., 2005). Increased accumulation of ADAMTS-5 in human OA cartilage compared to normal age-matched tissues has also been reported and supports a role for this enzyme in the pathogenesis of human OA (Plaas et al., 2007).
Figure 6: Zones of articular cartilage

From http://www.chelationtherapyonline.com/articles/p179.htm

S = superficial zone; M = middle zone; D = deep zone

From: http://www.nomranmarcusmd.com/research.html
Assessment of Cartilage Degeneration

Several in vivo techniques have been developed to diagnose and monitor the progression of cartilage degeneration. Radiographs are commonly taken, but they are unable to delineate cartilage directly because of lack of sensitivity to detect soft tissue contrast. They are commonly used to measure joint space width, an indirect assessment of cartilage thickness (Scott et al., 2007). Arthroscopy allows a direct view of cartilage and is considered the most reliable method for cartilage assessment (Lee et al., 2007). However, it is an invasive procedure potentially leading to post-surgical complications of infection and unintended joint injury. In addition, arthroscopy is expensive making it unsuitable as a global evaluative technique (Forssblad et al., 2004). Non-invasive imaging methods include computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography (US). CT, with and without contrast, produces images in the transverse plane (perpendicular to the direction of weight bearing), but cartilage over the weight bearing area of the knee cannot be directly viewed (Mackay et al., 2006). Current MRI techniques allow measurement of cartilage thickness (Burstein and Gray, 2006). They provide detailed images of tissue structure regardless of the plane by measuring levels of metabolites in cartilage and achieve superior soft tissue contrast compared to CT. MRI is able to detect cartilage abnormalities and meniscal injuries with the same resolution and clarity as arthroscopy (Hyusse and Verstraete, 2007). MRI can also be enhanced by injection of ionic gadolinium into the joint (Roos and Dahlberg, 2005). Healthy cartilage which is rich in negatively charged aggrecan repels the contrast while
degenerating cartilage with substantial aggrecan loss shows signal enhancement because it does not repel the contrast (Roos and Dahlberg, 2005). The usefulness of MRI in detecting and classifying cartilage degradation may also be improved by the development of on-line databases, such as morphological atlases of knee cartilage, and using shape indices as a means of classification (Tameem et al., 2007). Ultrasound correlates significantly with histological grading and continues to be studied to determine if it provides a non-invasive, cost-effective technique for accurately assessing cartilage quality (Lee et al., 2007).

Histological Grading of Cartilage in Human OA and in Animal Models
Cartilage tissue taken in animal studies or post-surgically in human studies can be graded based on its macroscopic and microscopic appearance. Noted pathological features include surface roughening, fissure development, delamination of the surface, cavity formation, tissue fragmentation, and fibrocartilage growth (Pritzker et al., 2006). A macroscopic classification system grades OA as Grades I-IV based on qualitative descriptions of the texture of the cartilage surface, the size of cartilage lesions, and changes in the subchondral bone (Collins et al., 1949). A 14-point microscopic grading system using histological stains, such as Safranin O with light green counterstain, is based on cellular changes, presence of Safranin O cartilage matrix staining, and changes such as erosion and vessel penetration. The Osteoarthritis Research Society International (OARSI) system is a 24-point score using six grades (based on
depth of progression into the cartilage) and/or four stages (based on percentage of involvement; Pritzker et al., 2006).

India ink wash of the joint surfaces allows further observation of cartilage surfaces and provides a means of quantifying the degradation based on the amount of India ink present (Chang et al., 1997; Kobayashi et al., 2000; Lewis et al., 2005; Stoker et al., 2006). Applying India ink highlights surface abnormalities of the articular cartilage. The India ink carbon particles (diameter 40-100 nm; Madsen et al., 1992) do not enter normal articular cartilage due to its small average pore size of ~6 nm (Maroudas, 1979) but does become entrapped in surface irregularities and adhere to the fibrillated cartilage (Meachim et al., 1972) thus appearing as darkly stained areas.

Clinical Treatment for Osteoarthritis

The current treatment modalities for OA are largely intervention-based, as they address symptoms only. Disease “cures” have not been successfully developed. Therapeutic approaches range from psychosocial, pharmacological, and physical therapy interventions to surgical replacements of degenerated joints. Effective long-term management of the disease proposes involvement of a sequential approach, the details of which are based on disease severity at the time of diagnosis. Such treatment strategies ideally begin with information and education at the earliest stages of disease diagnosis. This is followed by self help interventions (including analgesics) then progresses to simple [non-steroidal anti-inflammatory (NSAID) medications, corticosteroids, and physical and
occupational therapy] and advanced (injections) non-surgical interventions.

Finally, application of surgical procedures may be warranted (Figure 7). These treatment interventions and studies that have evaluated their efficacy are reviewed briefly in the following sections

Psychosocial Interventions

Psychosocial interventions such as patient education techniques and lifestyle modifications are commonly used in other chronic diseases such as diabetes (Delamater et al., 2001), obesity (Cresco et al., 2007), and cardiovascular (Patel and Adams, 2008) and neurodegenerative (Martial and Donahue, 2006) disorders. Similar approaches have been developed for OA and are utilized in many parts of the world in the treatment of OA. An education self help program was compared with “care as usual” in 273 persons with hip and/or knee OA to determine the effects on pain, other complaints (such as stiffness and loss of movement), and functional limitations. The patient group using the self help program reported decreased pain and improved function when compared to the “care as usual” controls as early as three months after starting. They reported improvements throughout a 21 month follow-up (Heats et al., 2005). Another study investigated the effects of coping skills training on
Figure 7: Sequential pyramidal approach to OA management

(From: Dieppe and Lohmander, 2005)
descending modulation of nocioception via the nociceptive flexion reflex (NFR) in persons with knee OA. The NFR results in withdrawing from a noxious stimulus mediated along the A-delta and C fibers which extend to the dorsal horn spinal neurons where the signal is modulated. The NFR threshold is correlated with subjective pain threshold. The intervention significantly increased NFR thresholds and decreased pain ratings (Emery et al., 2006).

*Educational interventions* such as therapeutic education and functional readaptation (TEFR) have also been tested for positive effects on health related quality of life. For example, a study of persons with knee OA on a wait list for total knee replacement comparing TEFR plus pharmacological treatment to pharmacological treatment alone found significant improvements in the Western Ontario and MacMaster's University Osteoarthritis Index (WOMAC) function score, pain, and physical function as measured by the Short Form Health Survey General Questionnaire (SF-36; Nunez et al., 2006).

In another study the effects of the Arthritis Self Management Program (Lorig et al., 1985) and the Chronic Disease Self Management Program (Lorig et al., 1999) on quality of life, health behaviors, self efficacy, and health care utilization was assessed. Both intervention programs demonstrated positive outcomes in all measures. However, the arthritis-specific program had better results at earlier time points than did the general chronic disease program (Lorig et al., 2005).

Specific *lifestyle modifications*, such as weight loss, have been shown to be efficacious in some persons with OA. One study involving a group of women
with a BMI < 25 showed that neither weight loss nor weight gain affected the risk of knee OA later in life. In a second group of women with BMI > 25, a weight loss of 12 pounds significantly lowered (>50%) the rate of knee OA. Further, in the group with BMI ≥25 weight gain was associated with an increased rate of knee OA later in life (Felson, 1990),

Similar findings were reported in a study of women who experienced knee pain and were overweight. An average weight loss of 15 pounds using a six month diet and walking intervention resulted in improved knee pain, decreased lower extremity disability, better vO₂ maximum, and greater six minute walk distance compared to baseline (Martin et al., 1996). A study of older adults (men and women aged 60 and older) with obesity and symptomatic knee OA reported that diet plus exercise and exercise alone led to decreased knee pain and less disability after the six month interventions (Messier et al., 2004). Furthermore, individuals in the diet plus exercise group lost 15 pounds more than those in the exercise alone group and had better improvements in knee-related disability.

Pharmacological Interventions

Oral Medications

Acetaminophen has been used for decades to treat symptoms of OA. Studies on less than 60 subjects for 6 weeks or less published in the 1980s and 1990s reported acetaminophen was better than placebo for pain relief in persons with OA (Amadio and Cummings, 1983; Zoppi et al., 1995). Due to its excellent safety record, large clinical trials which found a benefit of acetaminophen over
placebo (Miceli-Richard et al., 2004; Pincus et al., 2004), and meta-analyses showing a small effect size (Neame et al., 2004; Zhang et al., 2004), acetaminophen is still recommended as a first line treatment for pain associated with OA.

NSAIDS (ibuprofen, naproxen, nabumetone) are generally effective for pain relief in persons with OA. However, they may induce gastrointestinal or other complications (such as electrolyte imbalance, dizziness, and increased blood pressure) associated with long term use (Richy et al, 2004). NSAIDS have been reported to be marginally better than acetaminophen for pain relief (Eccles et al., 1998; Zhang et al., 2004), but little or no difference has been reported between formulations (Gotzsshe 2003; Scott et al., 2003). Given the higher rate of adverse effects associated with NSAIDS, they are added only if acetaminophen does not sufficiently relieve symptoms.

To address the issue of gastrointestinal side effects, cyclo-oxygenase 2 (COX-2) selective inhibitors were introduced as a new generation of NSAIDS. While studies demonstrated effectiveness in pain relief, the moderately selective COX inhibitors did not have better gastrointestinal safety (Ju’ni et al., 2002) and the highly selective COX inhibitors led to increased cardiovascular adverse events (Ju’ni et al., 2004).

Injectable Agents

Pharmacologic agents can also be delivered directly into the affected joint directly via an intra-articular injection. For example, corticosteroid injections for
short term pain relief during “inflammatory flares” of OA have been used to assist with physical therapy (Arroll and Goodyear-Smith, 2004). A meta-analysis of randomized controlled trials showed 1-4 weeks of pain relief after intra-articular injections of corticosteroids into OA knee joints (Arroll and Goodyear-Smith, 2004). However, a poor understanding of the detrimental effects of corticosteroid injections on the metabolic and structural integrity of joint tissues impedes on their usefulness as safe and reliable therapeutics (Divine et al., 2006).

A large number of studies have shown that intra-articular injections of HA decrease pain and improve functional outcomes in knee OA (Altman and Moskowitz, 1998; Huskisson and Donnelly, 1999; Kotz and Kolarz, 1999; Leardini, et al., 1987; Puhl et al., 1993; Wobig et al., 1998). HA is a high molecular weight polysaccharide composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine. It is produced by all cells in the body and secreted into the extracellular space with molecular weight s from 1-7 million Daltons. In articular joints, Type B cells in the synovial lining synthesize and secrete HA into the synovial fluid (Laurent et al., 1996) at a steady state concentration of 2-4 mg/ml (Balazs and Denlinger, 1993). Its high viscoelastic properties provide lubrication during slow joint movements and shock absorption during rapid joint movements (Brandt et al., 2000). Studies in the 1980s reported that HA concentration and size were reduced in synovial fluid collected from persons with OA of the knee (Dahl et al., 1985). Similar findings were reported in human temporomandibular joint disease as well (Takahashi et al., 2004). This led to postulation that intra-articular injection of HA may act to restore the
viscoelasticity and the lubricating functions in an HA-depleted joint (Balazs and Denlinger, 1993).

HA can also exert regulatory effects on cells through interaction with a range of HA receptors (CD44, toll-like receptors, LYVE-1, stabilin) (Nehls and Hayen, 2000). Such receptor interactions may lead to downstream inhibition of prostaglandin E$_2$ production (Yasui et al., 1992) and also provide protection against free radical cytotoxicity (Presti and Scott, 1994), altered inflammatory cell adherence, proliferation, migration, and phagocytosis (Ghosh, 1994).

The potential disease-modifying effects of intra-articular injections of HA may also work through the cell modifying pathways. Pain relief after three to five weekly, intra-articular injections of HA is reported to last from six months to a year (Altman and Moskowitz, 1998; Arrich et al., 2005; Bellamy et al., 2006; Huskisson and Donnelly, 1999; Kotz and Kolarz, 1999; Leardini, et al., 1987; Lo et al, 2003; Modawal et al., 2005; Puhl et al., 1993; Wang et al., 2004; Wobig et al., 1998) This is longer than its half-life (18-24 hours) within the joint (Kotz and Kolarz, 1999).

In vivo studies of human synovial fluid demonstrated decreased numbers of activated macrophages and lymphocytes (Corrado et al., 1995) and decreased synovial effusion (Dougados et al., 1993) with HA injections. HA may therefore have a local or systemic anti-inflammatory effect. Addition of HA to human articular cartilage explants ex vivo showed inhibition of IL-1β stimulated production of MMPs like MMP-1, MMP-3 and MMP-13 (Julovi et al., 2004). Addition of HA to fibroblast-like synoviocytes resulted in decreased expression of
ADAMTS-5 mRNA and this was accompanied by down-regulation of TNF-α, IL-8, and inducible nitric oxide synthase (iNOS) (Wang et al., 2006).

It should be noted that HA is also used clinically to prevent post-surgical adhesion development (Urman et al., 1991) in abdominal (Tsai et al., 2005) and ophthalmic (Arshinoff et al., 2002) surgery. In addition, increased concentration of HA in fetal wounds is one of the reasons for scarless wound healing (Mast et al., 1992).

The wound healing actions of HA have been attributed to a pro-inflammatory effect necessary for the granulation, re-epithelialization, and remodeling stages of wound healing. Its cellular effect enhances pro-inflammatory cell infiltration (Wisniewski et al., 1996), and increases local production of TNF-α, IL-1β, and IL-8 (Kobayashi and Terao, 1997). In addition HA can also augment the granulation phase of healing through enhancing cell migration (Ellis and Schor, 1996) and angiogenesis at the site of tissue damage (Deed et al., 1997).

In summary, the symptom-modifying effect of intra-articular HA in persons with OA may alter synovial cell proliferation, differentiation, and apoptosis and secondarily inhibit expression of pro-inflammatory signaling molecules and proteases in the joint that eventually lead to matrix destruction, as opposed to repair, in an OA joint (Goldberg and Buckwalter, 2005).
Physical Therapy Interventions

Physical therapy for the treatment of OA aims to correct joint biomechanics and provide symptom relief via orthotics and bracing, modalities, and exercise. The overall goal is to maximize functional capabilities while minimizing pain and trauma to soft tissues, bone, and cartilage surfaces (American Physical Therapy Association, 2003). These interventions are rarely used in isolation, but instead are used in conjunction with psychosocial and pharmacologic interventions.

Orthotics and Bracing

Orthotics and braces can change joint biomechanics and redistribute joint forces. The most frequently used orthotic in OA treatment is a lateral wedge which places the ankle and foot in less supination resulting in decreased stress on the medial compartment of the knee. Outcomes from studies using orthotic interventions vary depending on whether or not there was standardization of shoes in which the orthotics were inserted. Using lateral wedge insoles in a two-year study with persons with knee OA led to neither symptomatic (change in WOMAC scores, need for injections or lavage, or self-assessed activity) nor structural (joint space width) improvements, but a decreased number of days of NSAID usage was reported (Pharm et al., 2004). A four week intervention of lateral wedge insoles and standardized shoes gave significant improvements in WOMAC pain, stiffness, and function scores (Fang et al., 2006). A third study using an eight week intervention with elastic subtalar strapping reported...
radiographically detected decreases in femoritibial angle. Significant improvement in pain during bed rest with full knee extension, upon getting up from a seated position, and after getting up, as well as increased maximum distance walked, and aggregate severity score were also reported (Toda and Segal, 2002).

*Knee bracing* has also been utilized in an attempt to correct joint biomechanics at the knee and thereby reduce unusual forces at this joint. Indeed, knee valgus bracing improved disease-specific quality of life and function (Scott et al., 2003), and taping the patella in a more medial orientation also resulted in reduced knee pain (Cushnaghan et al.1994).

**Modalities**

Physical therapy modalities for the treatment of OA are used to decrease pain and inflammation (Brousseau et al., 2004). They include thermal modalities and electrical stimulation. Thermal modalities are primarily in the form of hot packs, cold packs, and ice massage.

While a single application of ice packs placed on the anterior and posterior knee did not significantly affect edema, ten sessions of cold pack application resulted in a significant effect (Hecht, 1983). Hot pack application did not have this effect. Twenty minutes of ice massage five times a week for two weeks was also found to decrease pain and improve knee extension strength, range of motion, and time to walk 50 feet (Yurtkuran, 1999).
Electrical stimulation elicits muscle contraction via the placement of electrodes on the skin (American Physical Therapy Association, 2003). This can assist in muscle strengthening as well as improving blood flow to the treated area. It has been suggested that the presence of the electrical current can attract and repel the positively and negatively charged ions in the underlying tissues and these may regulate a variety of cellular responses including inflammation and pain pathways (Kloth, 2005).

Pulsed electrical stimulation has been shown to improve patient and physician global evaluation and patient reported pain and reduce NSAID use by ≥ 50% (Farr et al., 2006; Garland et al., 2007). Neuromuscular electrical stimulation has also been shown to decrease pain in persons with knee OA (Gaines, et al., 2004).

Exercise

Therapeutic exercise is any activity designed to improve joint and/or muscle performance (American Physical Therapy Association, 2003). It includes aerobic/cardiovascular exercise, range of motion and flexibility, resistance training, and functional exercise. Exercise has long been used as a treatment intervention for persons with OA as it decreases joint pain and stiffness and increases functional abilities (Ettinger et al., 1997). Appendix 3 summarizes OA-related exercise studies and their reported outcomes.
Aerobic Exercise

Aerobic exercises include walking programs or stationary bicycling, are performed for at least 20 minutes at a time, and involve active movement that increases the heart rate. While aerobic exercise utilizes muscle action, muscle strengthening is not the primary objective. Aerobic exercise is generally performed to improve fitness level by increasing the efficiency of the heart and lungs with increasing activity. However, aerobic exercise also results in movement of the joints, loading of the bones, and muscle use, all of which may impact knee OA.

A large number of studies using aerobic exercise for persons with knee OA are reported. They all show significant improvements in a variety of functional, psychosocial, and pain measures. These measures include timed chair rise, six minute walk test, (Mangione et al., 1999), medication use, walking distance, function as measured by the Arthritis Impact Measurement Scale (AIMS) physical activity subscale (Kovar et al., 1992), 50 feet walking time, depression, anxiety, physical activity (Minor et al., 1989), walking speed, disability scores (Penninx et al., 2002; Ettinger et al., 1997), depressive symptoms as measured by the Center for Epidemiological Studies—Depression Scale (Penninx et al., 2002), self-efficacy for stair climbing (Rejeski et al., 1998), aerobic capacity (Mangione et al., 1999; Minor et al., 1989), and pain (Ettinger et al., 1997; Kovar et al., 1992; Mangione et al., 1999; Penninx et al., 2002).
Aerobic exercise did not increase daily reported pain, indicating this repetitive lower extremity exercise does not exacerbate pain symptoms (Mangione et al., 1999). Moreover, one study suggested that continuation of aerobic exercise is necessary in order to maintain benefits as cessation lead to outcome reports that were not significantly different from the non-exercising control group (Sullivan et al., 1998).

Indeed, several professional panels and consensus groups advocate aerobic exercise for persons with OA. The American Geriatric Society (AGS) Panel on Exercise and Osteoarthritis recommends aerobic exercise three to four times a week for 20-30 minutes a day at a low to moderate intensity which is further defined as 50-75% of the maximum heart rate or a rating of perceived exertion (RPE) of 10-13 (AGS Panel on Exercise and Osteoarthritis, 2001). The American College of Rheumatology (ACR) workgroup recommends 30 minutes of moderate intensity aerobic exercise (50-70% of maximum heart rate) at least three times a week (Altman et al., 2000). The MOVE consensus and the European League Against Rheumatism (EULAR) also recommend the inclusion of aerobic exercise for persons with OA, but do not make any recommendations on intensity, frequency, or volume (Jordan et al., 2003; Roddy et al., 2005).

According to Bennell and Hinman, strengthening exercises appear superior to aerobic exercise for persons with knee OA in improving physical symptoms, such as pain, whereas aerobic exercise may be more effective for functional outcomes over the longer term (Bennell and Hinman, 2005). However, a systematic review of 13 randomized controlled trials found no difference
between aerobic and strengthening exercise programs in their ability to reduce pain and disability in the same population (Roddy et al., 2006).

Since aerobic exercise has components of the other types of exercise (joint motion as in range of motion/flexibility exercise, strengthening to some degree as in resistance training, and activities of daily living as in functional exercise) investigating the benefits of aerobic exercise may provide a broad picture of the effect exercise can have on OA.

Range of Motion/Flexibility Exercises

Range of motion and flexibility exercises involve movement of extremities without resistance as well as stretching exercises aimed at increasing muscle length and flexibility to increase the range of motion within a joint. There are no studies specifically evaluating the effect of range of motion/flexibility exercises on persons with OA. Instead, these exercises are typically combined with other types of exercise (aerobic, resistance, and functional) for individuals with OA (Minor et al., 1989)

Resistance Exercises

Resistance exercises for OA involve moving a body part against an external force. This force ranges from bodyweight to low intensity (less than 20% of the maximum load a person can move through the full arc of movement) to high intensity (up to 85% of the maximum load a person can move through the
full arc of movement). Examples of such exercises are tightening the quadriceps muscle with the knee extended, standing up from a chair, using a knee extension or leg curl machine, and extending the knee in a seated position with weights around the ankle are all examples of resistance exercise. The amount of resistance is quantified by determining the maximum force an individual can generate or maximum load an individual can move during the exercise. Resistance exercises strengthen the muscles around an affected joint and thereby may improve muscle balance and joint biomechanics lessening stress on the joint tissues. In addition, resistance exercises result in joint movement and bone loading which may impact knee OA.

Resistance exercises have shown positive benefits for persons with OA in the following outcome categories: increased strength (Ettinger et al., 1997; Eyigor et al., 2004; Gur et al., 2002; Penninx et al., 2002; Schilke et al., 1996; Topp et al., 2002; Topp et al., 2005), decreased pain (Ettinger et al., 1997; Eyigor et al., 2004; Gur et al., 2002; Penninx et al., 2002; Schilke et al., 1996; Topp et al., 2002), decreased stiffness, and arthritis activity as measured by the Osteoarthritis Severity Index (OASI) and the AIMS (Schilke et al., 1996), improved function (Ettinger et al., 1997; Eyigor et al., 2004; Gur et al., 2002; Penninx et al., 2002; Schilke et al., 1996; Topp et al., 2002; Topp et al., 2005), less disability, decreased depressive symptoms (Ettinger et al., 1997; Penninx et al., 2002), decreased disease severity, and improved WOMAC, SF-36, and AIMS-2 scores (Eyigor et al., 2004).
Improving muscle strength, especially of the quadriceps, may be very important in preventing disease progression. Slemenda et al., found that women with stronger quadriceps had a reduced risk of developing radiographic evidence of knee OA as compared to those with weaker quadriceps (Slemenda et al., 1998). This finding was confirmed by Hootman et al, who looked at 3081 community dwelling adults without OA and found that women with moderate to high isokinetic quadriceps strength had a 55% reduced risk of developing hip OA and a 64% reduced risk of developing knee OA (Hootman et al., 2004). A strong quadriceps muscle may provide joint protection and decrease abnormal wear on the cartilage surfaces.

Increased hip abduction strength is also associated with decreased knee OA progression (Chang et al., 2004). Hip abductors originate on the pelvis above the hip joint and attach on the lateral leg just below the knee. As a result, they have a stabilizing effect on the pelvis and influence toe out angle during gait minimizing abnormal stress on the knee. Conversely, increased hip adduction strength is associated with increased disease progression and severity in the knee (Yamada et al., 2001) indicating that perhaps this muscle group should not be subject to strengthening exercises in persons with OA.

Although lower extremity muscle strengthening is beneficial in most persons with OA, the benefits of simply improving strength may be compromised by the enhanced quadriceps strength which in itself has been associated with increased disease progression in persons with malaligned or lax knees (Sharma
et al., 2003). Thus a careful screening of patients prior to strengthening exercise and appropriate modification of exercises is recommended to prevent joint trauma as a result of malalignment or laxity.

While some studies have been conducted to determine the most efficacious type of resistance training (isometric, isotonic, isokinetic), this area is still largely unstudied (Eyigor et al., 2004; Gur et al., 2002; Topp et al., 2002). In addition, evidence does not exist regarding the most appropriate load at which to perform resistance exercise in order to get the best gains for persons with OA.

Water-Based Exercise

Studies comparing water-based exercise to land-based exercise find functional gains achieved with both interventions. However, persons in the water-based program had better improvement in pain (Cochrane et al., 2005), walking distance and physical component on SF-12 (Foley et al., 2003) and persons in the land-based program had better improvements in strength, walking speed, and self-efficacy (Foley et al., 2003).

Functional Exercise

Functional exercises include activities, such as getting up from a chair and climbing stairs. If resistance is added to the activities, they are considered resistance exercises as in the case where an individual wears a weighted belt or vest while performing sit to stand or stair climbing. If the duration of the activity is sustained, it is considered aerobic exercise, such as climbing a flight of stairs five times.
Their use has been found to significantly improve times for performing activities of daily living, isokinetic quadriceps muscle strength, WOMAC scores, stair climbing, walking time, (Diracoglu e al., 2005; Lin et al., 2005) and proprioceptive sensation (Diracoglu e al., 2005).

Mode of Action of Exercise

Despite increasing consensus and evidence for exercise as an effective intervention in OA progression, the underlying physiologic mechanisms have not been determined. This is most likely due to the complex and pleiotropic effects of such a therapy on the physiology of the organism.

A commonly held view is that exercise improves joint biomechanics by restoring muscular balance and strengthening supporting structures around the joint, resulting in diminished stress and structural trauma to the cartilage. In vivo biomechanical studies showed that exercise might influence the integrity of muscle tissue. Thus, muscular balance and strengthening has been shown to diminish stress and structural trauma to the soft tissues (Hootman et al., 2004; Slemenda et al., 1998).

In vitro studies using tissue explants and isolated cells have revealed mechano-sensitive pathways, such as mitogen activated protein kinase and gene expression pathways for both degenerative and reparative actions of chondrocytes (Fitzgerald et al., 2006; Lee et al., 2005; Sah et al., 1989). In vitro studies of mechanical strain on chondrocytes show that low magnitude
mechanical strain on cartilage suppresses IL-1 and TNF alpha (both pro-inflammatory cytokines) and up regulates proteoglycan and collagen synthesis. However, high magnitude mechanical strain on cartilage is pro-inflammatory, initiating cartilage destruction and inhibiting matrix synthesis. (Deschner et al., 2003) This suggests a dose-response relationship between exercise and collagen synthesis in humans as well. However, what constitutes low magnitude and high magnitude mechanical strain with exercise is not clear, nor is the difference in response in humans with shear forces alone versus compressive forces alone versus a combination of forces.

The extent to which these are mechanistically related to events in the whole joint and in combination with soluble mediators (cytokines and growth factors) remains to be fully defined. Moreover, it is not entirely understood how the complex function of joint movement translates into specific types of biophysical signals such as compression, shear, fluid flow and streaming potentials (Fitzgerald et al., 2006; Gray et al., 1989; Kerin et al., 2002; Kim et al., 1995; Lee et al., 2005; Sah et al., 1989). The development of a simplified in vivo animal model of mechanical overuse and detailed cell and biochemical examination of the ensuing cartilage and bone response will aid greatly to this area of research.

The availability of high resolution imaging on tissue structures, such as delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC), an in vivo technique that relies on the principle of the negatively charged contrast agent distributing inversely to the negatively charged
glycosaminoglycans (GAGs) in the cartilage shows promising approaches to evaluate the effect of exercise on structural and biochemical changes in joint tissues such as cartilage (Link et al., 2006; Roos and Dahlberg, 2005; Tiderius et al., 2004). Roos et al. utilized dGEMRIC to investigate the effect of moderate exercise on knee cartilage quality and found that performing aerobic and weight bearing strengthening exercises three times a week for 4 months resulted in a significant improvement in cartilage quality as demonstrated by increased GAG content. This correlated with change in the Knee Osteoarthritis Outcomes Survey (KOOS) indicating an improvement in clinical status as well (Roos et al., 2005).

Physical exercise may also affect inflammation and the immune response. A study of 274 persons with knee OA participating in an exercise and nutrition program looked at serum levels of inflammatory markers including IL-6, CRP, TNF-α, and the soluble receptors (sR) IL-6sR, IL-2sR, TNF-sR1 and TNF-sR2. Higher serum levels of TNF-sR1 and TNF-sR2 (soluble receptors for a pro-inflammatory cytokine) were associated with lower physical function (Penninx et al., 2004). Further, these persons had more pain and stiffness and more reported physical disability as well as slower walking speeds and worse radiographic scores.

Isometric exercises may induce a beneficial inflammatory response in the joint tissue. For example, studies have shown that isometric exercises result in increased molecular weight of hyaluronan, increased viscosity of joint fluid, and decreased chondroitin sulfate concentration (Miyaguchi et al., 2003). Further, a
rise in serum HA, a marker of acute inflammation (Elliott et al., 2005), was noted with physical activity in persons with OA (Criscione et al., 2005).

Cell Based Therapies

Cartilage lesions have little capacity to spontaneously heal (Buckwalter and Mankin, 1997). As a result, cell-based approaches have been developed to assist in repairing cartilage defects. With these approaches, cells are manipulated in vitro then implanted in the cartilage defect (Richter, 2007). Mesenchymal stem cells have been used for this purpose because they can be expanded by in vitro culture and still maintain their multilineage potential (DeBari, 2006). Cartilage cells from autologous cartilage elsewhere in the body have also been removed, cultured, then implanted into the damaged area (Brittberg et al., 1994). This type of therapy may be enhanced by use of resorbable biomaterials for scaffolds into which chondrocytes are seeded and cultured, prior to implantation (Marlovits, et al., 2006). These methods have undergone extensive pre-clinical investigations using animal models of cartilage disease and defect, but are now being used in humans.

Surgical Interventions

Surgical interventions are generally implemented only after conservative measures have been unsuccessful (See Figure 2, Dieppe and Lohmander, 2005). They include both joint preserving (arthroscopy and osteotomy) and joint replacement techniques.
A commonly utilized surgical procedure for mild to moderate OA is arthroscopy. This technique can range from simple lavage and debridement to cartilage defect repair attempts and cartilage transplantation. Effectiveness of arthroscopic intervention is controversial (Chang et al., 1993; Gross et al., 1991; McEldowney and Weeker, 1995; McLaren et al., 1991; Moseley et al., 2002). If OA is contained to one side of the joint, a correction osteotomy is an option in order to transfer load-bearing from the affected to the non-affected region of the knee. A Cochrane review of 13 studies involving 693 persons concluded that osteotomy improves knee function and pain, but there is no evidence to support that osteotomy is more effective than conservative treatment (Brouwer et al., 2007).

Joint replacement techniques remove the pathological tissues and replace them with prostheses. While there are no randomized controlled trials comparing joint replacement to sham treatment or standard care, there are individual studies on sham interventions demonstrating a moderate effect on pain (Hrobjartsson and Gotzsche, 2001) and studies of joint replacement demonstrating a large effect on pain (Ashworth et al., 2002; Fortin et al., 2002).
Overview of Animal Models

Mechanistic studies of human disease progression and therapeutic efficacies have utilized animal models since the 1950s. Human OA is generally not diagnosed until it has progressed with pronounced pathological alterations in the joint leading to pain and radiographic changes. Further, obtaining diseased tissue prior to end-stage joint replacement surgery without potentially damaging the joint is not possible, and non-invasive methods of assessing changes in joint structure (i.e., MRI, radiographs) do not provide enough information on cellular and metabolic dynamics in the diseased joint. For these reasons, animal models of OA have been developed to provide a known cause of induction and time points of disease progression (Bendele, 2001).

Since injury is a common predisposing factor, most models involve surgically-induced insults in order to cause mechanical stress to the joint. Models have been developed in sheep (Cake et al., 2004), dogs (Mastberger et al., 2006), rabbits (Inouye et al., 1973), guinea pigs (Bendele et al., 1991), rats (Hayami et al., 2006; Laurent et al., 2006; Moore et al., 2005), and mice (Bendele, 2001). Direct comparison with age-matched, healthy tissue taken from animals of the same or similar genetic and environmental backgrounds can be readily obtained for radiographic, histologic, and biochemical evaluation methods. More recently murine (mouse) models have gained popularity with benefits of lower cost, homogeneity of genetic background, ability to inbreed, and
the capacity to obtain an increasing number of genetically modified strains. Creating surgically-induced injuries in mice has been difficult due to the small size of the joints and therefore requires microsurgical techniques (Kamekura et al., 2005).

Murine Models

Spontaneous OA

Age-onset development of OA occurs in STR, DBA/1, and C57 Black mouse strains. These “spontaneous” models may reveal mechanisms that underlie some forms of human OA (Yamamoto et al., 2005).

The highest incidence of this type of OA occurs in the STR/1N strain (Jay, 1951) and the related STR/ort strain (Sokoloff and Jay, 1956). Cartilage lesions are detected histologically as early as 8 weeks of age and appear at the insertion of the medial collateral ligament and the medial tibial plateau (Mason et al., 2001). Males have a higher incidence than females, and cartilage loss is most prevalent in the medial compartments of the knee joint. Interestingly, spontaneous obesity develops at three months of age in these mice, even when kept on a standard diet. Peak bone mass is reached at 12 weeks compared to 16 to 24 weeks in non-affected strains.

Other investigators using this mouse strain have reported high incidence of patella subluxation (Walton, 1979), decreased anterior cruciate ligament strength, soft tissue calcification (Evans et al., 1994) and increased biochemical turnover of collagen in cartilage (measured by gel zymography) all of which may
contribute to the spontaneous OA-like pathologies in these mice (Anderson-Mackenzie et al., 1999).

Histological evaluation of knee joints from STR/ort strain using TUNEL assays showed an increasing number of apoptotic cells in cartilage covering medial tibial and femoral bone with advanced histological lesions (Mistry et al., 2004). This finding may be consistent with preceding biomechanical or metabolic stress on the cartilage (Mason et al., 2001). In addition, cartilage of STR/ort mice has been reported to be deficient in extracellular superoxide dismutase, a reactive oxygen species (ROS) scavenger (Regan et al., 2005). This may predispose to oxidative damage to cells and accelerated apoptosis.

C57 Black mice are also susceptible to developing OA lesions in knee joints as a result of a genetic mutation with recessive Mendelian inheritance (Silberberg and Silberberg, 1960). Incidence and severity of OA based on histopathological cartilage damage assessed by hematoxyllin and eosin stain and decreased toludine blue staining (Yamamoto et al., 2005) increases with age in both males and females from 20% at 2 months to 80% at 16 months Cartilage erosion appeared in the subchondral layers of cartilage Decreased collagen content and fibril alignment as well as decreased proteoglycan contents accompanied the cartilage destruction (Yamamoto et al., 2005).

The development of OA at relatively predictable time points would render these “spontaneous” models appropriate for the evaluation of therapeutic interventions for disease progression. However, they have not been widely used
mainly due to the need to house and maintain these animals for up to a year which increases the cost of experimentation.

Surgically-Induced OA

Post-trauma models of OA models are typically induced surgically to create joint instability and have been applied in large animals, such as sheep (Cake et al., 2004), dogs (Mastberger et al., 2006), rabbits (Inouye et al., 1973), guinea pigs (Bendele et al., 1991), and rats (Hayami et al., 2006; Laurent et al., 2006; Moore et al., 2005). The mouse presents a challenge for surgically-induced OA because of its small size (Figure 9a) However, microsurgical techniques for meniscal and ligament destabilizations have been successfully utilized to induce OA-like joint changes in the knee (Kamekura et al., 2005). MCL transection and/or complete or partial meniscectomy are the most commonly used techniques, but other ligaments including the PCL, LCL, or ACL have also been transected (Kamekura et al., 2005). For example, MCL transection and partial medial meniscectomy lead to development of mild cartilage lesions (fibrillations and loss of superficial flattened cell layer) on the medial plateau in ~50% of treated mice by 2 weeks post surgery, and by 3-4 weeks all mice with this injury had developed OA-like changes (Clements et al., 2003).

The various surgical procedures are now typically classified on the severity and direction of instability: a) severe, after transection of all ligaments, including the patellar ligament, and removal of both menisci; b) moderate, after
ACL transection and medial menisectomy; c) mild, after ACL transaction; and d) medial, after MCL transection and medial menisectomy (Kamekura et al., 2005). While many of these changes may be consistent with joint changes seen in humans after ligament and/or meniscal injury, the medial model most closely resembles changes seen in the majority of persons with OA. However, the severe, moderate, and mild models may be helpful in investigating the development and progression of certain subtypes of OA.

Surgical models have been widely used by the pharmaceutical industry for pre-clinical studies on disease modifying OA drug development because of the rapid, reproducible changes that result. However, several limitations need to be considered. Firstly, OA is not always the result of significant trauma to the joint. While some patients with OA can pinpoint specific injuries in which ligaments or menisci were injured, many cannot. Hence OA may often be the result of cumulative microtrauma which is not well represented by surgically-induced models. Secondly, in surgically-induced models, cartilage damage occurs in a relatively short time (1-2 weeks post surgery), whereas progression of human OA occurs over the course of years. In addition, when humans sustain such joint injuries it is rare for them to continue loading the joint. Most undergo surgical repair in an attempt to restore joint stability. In instances where stability cannot be successfully restored, activity is severely limited and therefore, joint loading is minimized. Thirdly, surgical models have only limited use for evaluation of therapies because the amount of trauma induced in the absence of surgical repair does not allow “normal” joint loading and leads to continued joint damage.
even in the presence of interventions. Fourthly, surgeries can easily result in direct damage to other joint structures, such as synovium and cartilage, at the time of surgery that may initiate additional wound healing pathways (including scarring) that are not normally part of the human OA pathology. Fifthly, the biomechanics of joint loading in the mouse is quite different from that in the human and may not provide accurate information regarding the effects of joint instability.

Manipulation of Cartilage Specific Genes

Genetic models provide insight into possible causative factors and generate strains that consistently develop OA (Clements et al., 2003; de Hooge et al., 2005; Giancotti and Rusoslahti, 1999; Saamanen et al., 2000; Xu et al., 2003; Zemmyo et al., 2003). Mutations known to make humans susceptible to OA involve collagens II (Vikkula et al., 1994), IX (Olsen, 1997), and XI (Jacenko and Olsen, 1995). As a result, many of the genetic models focus around these candidate genes.

Spontaneous single nucleotide deletion in the ColXIa1 gene (α1 chain of type XI collagen) in the cho/+ strain results in thickened collagen fibrils in the cartilage (Xu et al., 2003) and leads to destruction of cartilage and biochemical alterations, such as increased chondrocyte metabolism and increased proteoglycan degradation, similar to human OA (Tetlow et al., 2001).

Del 1 (+/-) strain has a deletion mutation in the transgene coding for type II collagen (Saamanen et al., 2000). These mice produce shortened pro-alpha 1
chains of collagen II and develop superficial fibrillation of cartilage at 12 weeks of age. This progresses to cartilage erosion, meniscal degeneration, bony sclerosis, and exposure of subchondral bone (Saamanen et al., 2007).

Deletion of an alpha 1 integrin, a transmembrane protein that binds components of the extracellular matrix and may be involved in cell activation, differentiation, proliferation, and survival (Giancotti and Rusoslahti, 1999) results in synovial hyperplasia, increased cellularity and apoptotic cells in the cartilage, and finally severe cartilage degeneration (Zemmyo et al., 2003).

The primary benefit of the genetic models is the known molecular etiology and the controlled severity and incidence of OA pathogenesis. However, limitations exist. These mice can be very difficult to breed and may need specialized housing, both increasing the cost of experiment. Further, it is not known if the gene is directly involved in OA or whether developmental changes due to the deletion predisposes an individual animal to OA. For example, many of these models develop skeletal deformations such as chondroplasias. These models are not readily amenable to treatment interventions unless conditional knock-outs are generated that display the gene abnormality only during adulthood or upon induction of the disease. In addition disease progression may not always mimic that which occurs in human disease because of a more selective activation of pathogenetic pathways by genetic alterations and limited representation of the many facets of OA development and progression (Figure 1) may result.
Chemical

OA-like changes can also be induced in joints by injection of noxious agents such as, quinolone antibiotics (Christ et al., 1988), iodoacetate (Guingamp et al., 1997; Janusz et al., 2001), collagenase (Blom et al., 2004) and pro-inflammatory agents such as TGF-β1 (Van Beuningen et al., 2000).

Quinolone, an antibiotic that targets bacterial topoisomerase, causes articular cartilage degeneration in juvenile animals. These changes occur rapidly (within 24 hours) and include focal swelling, chondrocyte death, and blister-like lesions in the mid zone cartilage (Bendele, 2001). With time the cartilage surrounding these lesions also deteriorates, with fibrillation and proteoglycan loss similar to that seen in other models of OA (Bendele, 2001). While this model has been used in guinea pigs (Bendele et al., 1990) and dogs (Gough et al., 1992), it is rarely used because it affects only growth cartilage, but not articular cartilage, and its mechanism is not well understood (Simonin et al., 1999).

Bacterial collagenase induces osteophyte development at the periarticular margins followed by fibrosis and an influx of macrophages into the synovial lining. This model is representative of some of the early changes seen within OA, but does not reflect pathology seen in late stages of OA other than osteophyte formation (Blom et al., 2004).

Iodoacetate causes cartilage thinning and fibrillation (Janusz et al., 2001) as well as osteophyte development (Guingamp et al., 1997), but also produces hyperalgesia and allodynia within one week of injection (Fernihough et al., 2004). Because of the pain presentation, this method is primarily used to study pain.
The pain symptoms produced can be controlled by oral administration of Naproxen, rofecoxib, and acetaminophen which are also used for pain relief in persons with OA (Bove et al., 2003).

Intra-articular injections of TGF-β1, a multifunctional modifier of skeletal tissue growth (Heino et al., 2002; Yan et al., 2001), was first reported by Van Beuningen et al. to induce OA-like changes in mouse knees. After multiple (up to three) injections into the knee joint on alternate days, they reported osteophyte formation (Van Beuningen et al., 2000) and influx of macrophage-like cells in the synovial lining, which are important producers of cytokines and growth factors regulating mesenchymal cell activities (Rappolee and Werb, 1992). Recent studies using this model investigated the role of macrophages in osteophyte formation (van Lent et al., 2004) and the role of interleukin-1 in OA development (Blamey Davidson et al., 2005).

The primary benefit of this model is that OA-like changes can be initiated within the joint in a very short period of time (less than a week in some instances). Joint anatomy is maintained making it highly suitable to evaluating therapeutic intervention effects.

As is the case for the surgical models, the injection models require specialized, technical expertise to perform the targeted injections. The injection (needle) may damage tissues within the joint cavity leading to additional injury responses and substances may not be quantitatively delivered to the joint space.
Animal Models and Therapeutic Hyaluronan Injections

The effects of intra-articular injections of HA on OA have not been well studied in mice. However, some work has been done in this area on other animal models. Rabbits receiving five weekly HA injections one month after anterior cruciate ligament transection to induce OA demonstrated less severe cartilage damage than the non-HA injected controls (Amyl et al., 2003; Takahashi et al., 1999). In addition, a down-regulation of proteolysis enzymes such as MMP-3 and cytokines, such as IL-1β in the synovium was reported (Takahashi et al., 1999). This process appears to be partially mediated by CD44, a cell surface receptor for HA (Waddell et al., 2007). In another report, rabbits with anterior cruciate ligament transection that had five or ten weekly HA injections showed less disease progression. This could be improved by increasing the therapy to ten weekly injections (Amyl et al., 2003).

After bilateral partial medial meniscectomies in rabbits, the application of five weekly HA injections resulted in significantly lower nitric oxide production and decreased cell apoptosis in the central region of the meniscus (Kobayashi et al., 2002; Takahashi et al., 2000) as well as increased collagen remodeling in the peripheral region (Sonada et al., 2000)

Animal Models and Treadmill Exercise

There are limited studies using aerobic exercise with mice (Table 1). Although treadmill running has not been utilized in mice with OA it has been used
in non-OA mouse models. In a study of the effect of short term moderate exercise on tumor metastases C57Bl/6 mice ran for one hour a day for 6 days at a speed of 36 meters per minute (Murphy et al., 2004). G93A transgenic mice with a mutation causing the development of amyotrophic lateral sclerosis ran 30-45 minutes per day three to five times a week for eight weeks at speeds of 15-37 cm per second (Mahoney et al., 2004). The same mouse strain was used to demonstrate that regular exercise was associated with an increased lifespan after running the mice 30 minutes per day, five days a week at a speed of 21 cm per second for 10 weeks (Kirkinezos et al., 2003).

Lightfoot et al. studied aerobic capacity variations in ten inbred strains by running mice at 36-72 cm per second until the mice were unable to continue (Lightfoot et al., 2001). C57Bl mice ran an average of eight minutes on testing which is significantly lower than four other strains and places them on the lower end of the spectrum of aerobic capacities in the strains tested (Lightfoot et al., 2001).

Massett and Berk also investigated exercise training response in six inbred and hybrid mouse strains by running them at speeds of 25-32 cm per minute one hour per day, five days per week for four weeks (Massett and Berk, 2005). Subsequently exercise performance was assessed by running at speeds of 14-62 cm/sec until exhaustion. Endurance exercise performance of C57Bl mice was similar to Balb/c but significantly lower than other strains with only small improvements in endurance exercise performance (22-33% increase in distance) occurring after four weeks of training (Massett and Berk, 2005).
Finally, critical running speed (the speed that can be sustained for 40-60 minutes) was studied in three mouse strains by running at 30-85 cm per second until exhaustion (Billat et al., 2005). For C57Bl mice, critical speed was determined to be 20 cm per sec.

Maximum aerobic speed has been determined for mice and although it has been shown to vary slightly between mouse strains (Billat et al., 2005; Massett and Berk, 2005) it can be used to determine maximum oxygen uptake (vO₂ max), the maximum capacity to transport and utilize oxygen during incremental exercise (Baker and Gleeson, 1999). A percentage of vO₂ max is the best method utilized in prescribing exercise with 40-60% vO₂ max coinciding with low intensity, 60-80% coinciding with moderate intensity, and greater than 80% coinciding with high intensity (Swain et al., 2004). Using the formula vO₂ = 3.99 + 1.94v where v is the speed, low intensity aerobic exercise for the mouse at 50% vO₂ is achieved at approximately 14 cm/sec (Baker and Gleeson, 1999).

The effect of treadmill exercise on OA in mouse models has not been studied. Only voluntary wheel running in normal and collagen IIa1 knockout mice was reported (Lapvetalainen et al., 2002). Running did not affect OA development in normal mice but reduced incidence of spontaneous OA in the knockouts (Lapvetalainen et al., 2002).
<table>
<thead>
<tr>
<th>Study</th>
<th>Strain</th>
<th>Mode of Exercise</th>
<th>Effect Investigated</th>
<th>Outcome</th>
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</thead>
<tbody>
<tr>
<td>Lapvetalainen et al., 2002</td>
<td>Col 2a1 KO</td>
<td>Voluntary wheel running</td>
<td>OA development</td>
<td>Reduced OA development</td>
</tr>
<tr>
<td>Murphy et al., 2004</td>
<td>C57Bl/6</td>
<td>Treadmill running</td>
<td>Lung tumor metastases</td>
<td>Decreased metastases</td>
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<td>Mahoney et al., 2004</td>
<td>G93A</td>
<td>Treadmill running</td>
<td>ALS onset and survival with high intensity endurance training</td>
<td>No affect on onset of symptoms; faster decrease in motor performance and death in male mice only</td>
</tr>
<tr>
<td>Kirkinezos et al., 2003</td>
<td>G93A</td>
<td>Treadmill running</td>
<td>ALS lifespan with moderate intensity endurance training</td>
<td>Increased lifespan; males greater than females</td>
</tr>
<tr>
<td>Lightfoot et al., 2001</td>
<td>10 strains of inbred mice</td>
<td>Treadmill running</td>
<td>Interstrain variation in aerobic capacity</td>
<td>Strong genetic contribution to aerobic capacity in mice—Balb/c mice had 3 times greater aerobic capacity than C57/Bl6 mice</td>
</tr>
<tr>
<td>Massett and Berk, 2005</td>
<td>C57Black</td>
<td>Treadmill running</td>
<td>Exercise training response</td>
<td>22-33% increase in distance after 4 weeks of training</td>
</tr>
<tr>
<td>Billat et al., 2005</td>
<td>C57Black</td>
<td>Treadmill running</td>
<td>Critical running speed</td>
<td>Critical running speed is 20 cm/sec; $v_{0_2}$ max is approximately 8.0 ml O$_2$/g·h</td>
</tr>
</tbody>
</table>
Chapter Two
Specific Aims and Research Hypotheses

OA is a debilitating condition affecting over 21 million persons in the United States. This number is expected to rise in the coming decades. Current treatment approaches for OA focus on symptom modifying measures (i.e., pain relief) as disease modifying interventions do not currently exist. However, some of the interventions used to relieve and improve the symptoms of OA may actually have disease-modifying benefits. Two such non-surgical interventions for OA are intra-articular HA injections and exercise.

In order to effectively study the mechanistic aspects of such treatment options, an animal model of OA that is amenable for studying such intervention outcomes is needed. Mouse models of OA have become increasingly popular tools for this type of research due to rapidity, ease of disease assessment, cost-effectiveness, and availability of targeted gene knock-in and knock-out strains. The overall aims of this dissertation project were to: a) develop a rapidly progressing mouse model of knee OA that does not need surgical intervention or noxious chemical agents; and, b) test the response of the model to disease-modifying interventions such as HA injections or exercise. The data obtained from this study will provide novel information to refine and implement such interventions in studies using larger animals and human patients. They can also
be incorporated into educational materials for patient education in psychosocial and physical therapy interventions.

Research Hypotheses

1. Comprehensive OA-like changes in murine knee joints (synovitis, soft tissue fibrosis, osteophyte formation and cartilage and meniscus degeneration) can be induced by anabolic stimulation of joint tissues by intra-articular TGF-β1 followed by mechanical overuse of the activated joint.

2. Cartilage degeneration in the above model can be inhibited by therapeutic intervention during the anabolic stimulation phase, using intra-articular HA injections.

3. Cartilage degeneration in the above model can be decreased by four weeks of alternate day, low intensity aerobic exercise preceding the anabolic stimulation phase.

Specific Aims

The research was conducted under three specific aims:

Aim 1. Develop and characterize a progressive non-surgical model of knee OA in adult mice. C57Bl/10 male mice were subjected to two alternate day intra-articular injections of TGF-β1 into one knee joint, followed by high intensity treadmill running at 32 cm/sec for 13 days. Joint pathology was evaluated by radiographs, India ink cartilage grading, and histopathology. The validity of the
OA model was established by demonstrating resistance of ADAMTS 5-/- mice to cartilage degeneration.

**Aim 2.** Examine the capacity of intra-articular HA injection to inhibit progression of knee joint degeneration in the mouse model established under Specific Aim 1. High molecular weight HA (Supartz™, Clinical Grade) was injected intra-articularly during the anabolic /inflammatory phase of the model. Metabolic turnover routes of injected HA in the joint were examined by in vivo imaging of fluorescently labeled HA in the knee joint and by quantitative ELISA of HA in plasma. Joint pathology was evaluated by radiographs, India ink cartilage grading, and histopathology.

**Aim 3.** Examine the capacity of low intensity aerobic exercise to prevent onset and progression of joint degeneration in the mouse model established under Specific Aim 1. C57Bl/10 male mice underwent four weeks of alternate day, low intensity treadmill running at 14 cm/sec prior to inducing OA by TGF-β1 injection and mechanical overuse through high intensity treadmill running. Joint pathology was evaluated by radiographs and India ink cartilage grading.

Each specific aim and corresponding research hypothesis will be addressed in separate chapters following. The research design, materials, methods, results and analyses, and discussion and conclusions for the development of the model will be presented in chapter 3, the HA intervention in chapter 4, and the aerobic exercise intervention in chapter 5.
Chapter Three

Development of Mouse Model of Knee OA

All in vivo experimental protocols described below have been approved by the IACUC of Rush University, Medical Center, under Protocol 07-009.

Research Design (Figure 8 and Table 2)

Experimental groups:

1.1 Cage Control Day 5 (n=14): no TGF-β1 injections, cage activity, sacrificed day 5

2.1 Cage + TGF-β1 Day 5 (n=19): TGF-β1 injections, cage activity, sacrificed day 5

3.1 Cage + Sham Day 5 (n=19): BSA injections in lieu of TGF-β1 injections, cage activity, sacrificed day 5

4.1 Cage Control Day 18 (n=18): no TGF-β1 injections, cage activity, sacrificed day 18

5.1 Cage + TGF-β1 Day 18 (n=23): TGF-β1 injections, cage activity, sacrificed day 18
Figure 8: Research design for the development of a non-surgical mouse model of OA

Day 5

Overuse through high intensity treadmill running
• Speed = 32 cm/sec
• Duration = 20 minutes
• Frequency = daily x 13 days

Day 3

Mechanical Overuse

A

Day 1

TGF-β1 Injection Right Leg (2x 48 h interval)

Day 5

Cage

Day 18

Sacrifice

B

Day 1

TGF-β1 Injection Right Leg (2x 48 h interval)

Day 5

Sacrifice

Day 18

Sacrifice
6.1 Cage + Sham Day 18 (n=19): BSA injections in lieu of TGF-β1 injections, cage activity, sacrificed day 18

7.1 Mechanical Overuse Day 18 (n=20): no TGF-β1 injections, mechanical overuse via high intensity treadmill running, sacrificed day 18

8.1 Mechanical Overuse + TGF-β1 Day 18 (n=19): TGF-β1 injections, mechanical overuse via high intensity treadmill running, sacrificed day 18

9.1 Mechanical Overuse + Sham Day 18 (n=18): BSA injections in lieu of TGF-β1 injections, mechanical overuse via high intensity treadmill running, sacrificed day 18

In addition to C57Bl/10 mice, 129SvEv^Brd^ Agg 2/ mice (ADAMTS-5 Knockouts) and their controls (129S6/SvEv^Tac^) were used to determine if ADAMTS-5 plays a role in the cartilage changes. Only males 12-16 weeks of age were used.
Table 2: Mouse utilization for the induction of OA-like changes

<table>
<thead>
<tr>
<th>Strain</th>
<th>C57Bl/10 n=169</th>
<th>Agg 2-/- n=36</th>
<th>129S6/SvEv n=42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluation</td>
<td>H/E India ink and FACE</td>
<td>H/E India ink and FACE</td>
<td>H/E India ink and FACE</td>
</tr>
<tr>
<td>Cage Control Day 5</td>
<td>8/6</td>
<td>2/0</td>
<td>2/0</td>
</tr>
<tr>
<td>Cage + TGF-β1 Day 5</td>
<td>13/6</td>
<td>2/0</td>
<td>2/0</td>
</tr>
<tr>
<td>Cage + Sham Day 5</td>
<td>13/6</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Cage Control Day 18</td>
<td>12/6</td>
<td>2/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Cage + TGF-β1 Day 18</td>
<td>17/6</td>
<td>2/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Cage + Sham Day 18</td>
<td>13/6</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Mechanical Overuse Day 18</td>
<td>14/6</td>
<td>2/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Mechanical Overuse + TGF-β1 Day 18</td>
<td>13/6</td>
<td>2/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Mechanical Overuse + Sham Day 18</td>
<td>12/6</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>
Materials

TGF-β1 (PeproTech, Inc., Rocky Hill NJ); Bovine Serum Albumin (Sigma Aldrich, St. Louis MO); India ink (Speedball Arts Product Co., Statesville NC); ketamine (Phoenix Pharmaceutical Inc., St. Joseph MO); xylazine (Phoenix Pharmaceutical Inc., St. Joseph MO); injection needles (Tyco Healthcare Group, Mansfield MA); 5 mm and 3 mm micro dissection spring scissors (Roboz Surgical Instrument Co. Inc., Gaithersburg MD); Human TGF-β1 ELISA kit (Bender Med Systems, Burlingame CA); eppendorf tubes (DOT Scientific, Inc., Burton MI); heparin (Becton Dickinson Vacutainer Systems, Rutherford NJ); hematoxylin (Richard Allan Scientific, Kalamazoo MI); eosin (Richard Allan Scientific, Kalamazoo MI); HA ELISA materials were obtained and prepared as described (Li et al., 1989; Rayan et al., 1998; Thonar et al., 1992); FACE chemicals were obtained and prepared as described (Plaas et al., 2001).

General purpose, two-lane mouse treadmill with single belt construction and dividing wall outfitted with an electrical stimulus system composed of two shock grids with stimulus intensity (163 V at a range of 0-1.5 mA, for a max of 0.5 seconds per shock) and monitored by LED lamps to indicate which stimulus grid is active (Stoelting, Inc., Wood Dale IL). The manufacturer’s design of the electrical stimulus grid reflects special attention to avoid injuries to animals.
Methods

Mouse Breeding and Husbandry

The following strains of mice were used for experimentation: C57Bl/10 male mice procured from Jackson Laboratories or National Cancer Institute at 7-11 weeks of age and housed until they were 12 weeks old; 129S6/SvEvTac breeding pairs from Taconic; 129SvEvBrd Agg 2-/- (ADAMTS 5-/- exon 2 back crossed into the C57B./6 strain) breeding pairs obtained from Charles River Laboratories through Dr. Micky Tortorella and Dr. Anne-Marie Malfait (Pfizer, Inc.). Twelve week old 129S6/SvEvTac and 129SvEvBrd Agg 2-/- (ADAMTS 5-/-) mice used for experiments were obtained through an in-house breeding program. All mice were housed in polycarbonate shoebox rodent cage (five mice per cage) with corncob pelleted bedding and were given free access to food (Harlan Tekland Global Protein Rodent Diet) via wire bar lid and acidified water via water bottle and/or automated watering system. Animals were monitored for health on a daily basis and received cage changes every two weeks. For procedures requiring restraint, mice were held by the scruff of the neck with thumb and forefinger and the tail was secured between the fourth and fifth fingers. Intra-articular injections and blood collections were performed under general anesthesia consisted of a ketamine/xylazine cocktail (100mg/kg ketamine + 10 mg/kg xylazine) in sterile PBS delivered intraperitoneally via a 28G x ½ inch needle on 0.5 ml syringe. Upon termination of the protocol mice were humanely euthanatized via carbon dioxide followed by cervical dislocation to confirm death.
Intra-Articular TGF-β1 Injections

Twelve-week old adult male mice were anesthetized as described above, right legs shaved with enough border to prevent fur from contaminating the injection site and the knee joint area rinsed with dilute iodine solution. TGF-β1 (PeproTech, Inc., Rocky Hill, NJ) (200 ng in 6 µl of sterile PBS/0.1% purified BSA) or 6 µl BSA solution alone was injected with a 28G x ½ inch needle through the patellar ligament into intra-articular space of the knee joint (Figure 9). Animals were allowed to recover and then transferred back to their home cage. Injections were repeated after a 48 hour interval to achieve repeatedly high levels of this factor in the joint.
Figure 9: Site of needle entry for intra-articular injection of the knee between the femur and tibia just below the patella through the patella ligament and into the joint capsule. a) Mouse shown actual size; and b) right knee joint enlarged to show a more detailed injection site

a)

b)
Blood Collection and Plasma Preparation

Blood (5-7 drops for in vivo time points and terminal bleeds at time of sacrifice) was collected from the submandibular vein at baseline using a sterile 18G needle, two days post first TGF-β1 injection (day 3), two days post second TGF-β1 injection (day 5), one week post second TGF-β1 injection (day 12), and 14 days post second TGF-β1 injection (day 18). Prior to all blood draws anesthesia was administered as described previously. Blood was collected into heparinized, sterile Eppendorf tubes, cells removed by centrifugation at 13,000 rpm for ten minutes at room temperature, plasma supernatants removed and stored at -80º C until further analyses.

TGF-β1 Enzyme Linked Immunosorbance Assay (ELISA)

Twelve-week old adult male mice were divided into control and treatment groups. The treatment group (n = 6) received TGF-β1 injections as described above. The control group (n = 6) received intra-articular injections of BSA. Blood was drawn daily and prepared as described previously. Mouse TGF-β1 ELISA and human TGF-β1 ELISA was performed according to manufacturer’s directions (Bender Med Systems, Burlingame CA).

ELISA detection of circulation human TGF-β1 in the mouse was negative and consistent with the injected protein being utilized within the joint and/or cleared by the lymphatic system. Interestingly, plasma levels of
endogenous (mouse) TGF-β1 were elevated following each intra-articular TGF-
β1 injection (Figure 10). In comparison, intra-articular injection of BSA resulted in
a temporary decrease in endogenous plasma TGF-β1.
Figure 10: Endogenous (mouse) TGF-β1 concentrations for mice injected with human TGF-β1 and mice injected with BSA as measured by mouse TGF-β1 ELISA and expressed as a percentage of predose. Each time point represents two assays.
Mechanical Overuse Through High Intensity Treadmill Running

Treadmill running was initiated one day after the second TGF-β1 injection (Figure 11). The mice were familiarized with running on the treadmill for two days at speeds of 24-32 cm per second for 20 minutes per day. Strenuous treadmill running was started on day three and continued for 20 minutes per day for 13 days. Running speed was calculated as follows: using the formula \( \text{vO}_2\text{max} = 3.99 + 1.94v \) where \( v \) is the speed (km/hour) and \( \text{vO}_2\text{max} \) for C57Bl mice is 8.0 ml \( \text{O}_2/\text{g·hour} \), high intensity aerobic exercise for the mouse at approximately 80% \( \text{vO}_2 \) is achieved at a speed of 32 cm/second (Baker and Gleeson, 1999; Billat et al, 2005).

A daily record was kept for each animal that included: duration of the run (minutes), distance run (calculated from the run time and speed of the treadmill), and number and time of interactions with the shock grid. In addition, treadmill performance was measured by the amount of time each mouse stayed on the front half of the running treadmill (Figure 11).

Mice readily adapted to the daily treadmill running. However, if an animal refused to run continuously during the initial familiarization period, or up to 2 consecutive days during therapeutic exercise, it was excluded from further studies. No signs of pain or distress even at the early inflammatory stage after TGF-β1 injection were noted, excluding the need for analgesics.
Figure 11: Mechanical overuse through high intensity treadmill running
The C57Bl/10 mice ran 381.7 meters per day on average and the 129SvEvBrd Agg2-/- mice ran 379.2 meters per day on average. A difference was noted between C57Bl/10 TGF-β1 injected and non-injected mice in the time spent on the front half of the treadmill. Initially both groups spent an average of 19.5 minutes on the front half of the treadmill. The non-TGF-β1 injected mice continued to spend an average of at least 18 minutes on the front half of the treadmill throughout the 13 day period. However, by day 5 of running (day 9 of the protocol) the TGF-β1 injected mice spent an average of 18 minutes or less, and by day 17 they spent an average of 14.6 minutes. This difference between TGF-β1 injected and non-injected mice was not apparent in the 129SvEvBrd Agg2-/- (ADAMTS 5 KO) strain. However, the knockout mice did not run as well as the C57Bl/10 mice. The 129SvEvBrd Agg2-/- (ADAMTS 5 KO) mice came in contact with the shock panels more frequently and remained in contact with the shock panels for longer periods of time. The most notable difference was in the time spent on the front half of the treadmill. The C57Bl/10 mice ran on the front half of the treadmill (away from the shock panel) an average of 19.5 minutes while the 129SvEvBrd Agg2-/- (ADAMTS 5 KO) mice ran on the front half of the treadmill an average of 0.7 minutes. In addition, the 129SvEvBrd Agg2-/- (ADAMTS 5 KO) mice developed abrasions on their feet as a result frequent contact with the shock panel. These abrasions were treated with silvadene cream.
Tissue Harvesting

Hind limbs were harvested from euthanatized animals for histopathological evaluation via transection of the mid shaft of the femur. Fur, skin, and excess muscle tissue was removed, limbs placed in saline soaked gauze, and stored at -20° C evaluation as below.

Radiography

Animals were sacrificed on day 5 and day 18 and placed on a transparent platform with lower extremities in full knee extension in an anterior/posterior view and in a 90° flexed position in a medial/lateral view. Radiographs of the lower body were taken using the KODAK Molecular Image Station-In Vivo FX with Kodak Molecular Imaging Software with settings of 60 second x-ray exposures, no filters, f-stop 5.8, and a field of view of 40. Imaging allowed visualization of osteophyte formation and bony alignment.

India Ink Cartilage Surface Evaluation

Animals were sacrificed on day 5 and day 18, hind limbs harvested as described above, and tissues stored in saline soaked gauze at-20 degrees C. Prior to India ink staining joints were dissected under a Nikon SMZ1000 microscope as follows: 1) the patella ligament was transected and diagonal cuts along medial and lateral joint lines were made to allow the patella to be pulled superiorly away from the joint; 2) medial and lateral collateral ligaments were then transected allowing joint distraction and transection of the cruciate
ligaments; 3) remaining joint capsule was cut to allow full separation of the tibia and femur; 4) residual synovial tissue and menisci were removed from tibial plateaus and femoral condyles to allow full exposure of the cartilage surfaces. Cartilage surfaces were then rinsed with PBS and photographed with a Nikon SMZ 1000 camera on a dissecting microscope and SPOT Basic software version 3.5.9 for Windows.

India ink was applied to femoral condyles and tibial plateaus with a small brush and allowed to set for two minutes. Unbound ink was removed with saline washes (up to 30 seconds) and gently dabbing with a cotton-tipped applicator. Marked cartilage surfaces were re-photographed.

Images were viewed in Adobe Photoshop for scoring (Figure 12). Femoral and tibial surfaces were divided into four quadrants each and cartilage damage assessed in each quadrant by the following grading system (Kobayashi et al., 2000).

0 = Grade 1 (intact surface): surface normal in appearance and does not retain ink
1 = Grade 2 (minimal fibrillation): surface appeared normal before staining, but the India ink showed fibrillation
2 = Grade 3 (overt erosion): surface fibrillation was apparent before staining and areas retained ink as intense black patches
3 = Grade 4 (ulceration): loss of cartilage exposes underlying bone

Thus for each tibial or femoral surface a scoring range of 0-12 can be determined with higher scores denoting increased cartilage damage.
Cartilage scores for all four quadrants were analyzed individually then summed to obtain the overall cartilage score for each cartilage surface (right femur, left femur, right tibia, and left tibia). In addition, quadrants 1 and 2 were summed for the anterior surface score, quadrants 3 and 4 for the posterior score. Finally quadrants 1 and 3 were summed for the lateral cartilage score and quadrants 2 and 4 for the medial cartilage score (Figure 13).
Figure 12: Example grading of femoral and tibial cartilage surfaces after India ink staining

0 = normal cartilage
1 = minimal fibrillation
2 = overt erosion
3 = ulceration
Figure 13: Cartilage surfaces were divided into four quadrants. Anterior cartilage = quadrant 1 + quadrant 2; posterior cartilage = quadrant 3 + quadrant 4; lateral cartilage = quadrant 1 + quadrant 3; medial cartilage = quadrant 2 + quadrant 4.
Hematoxyllin/Eosin Histopathology

Hind limbs were dissected at the mid-femur, skin and muscle were removed prior to fixation in 10% neutral buffered formalin for 48 hours, and specimens were decalcified for 2 weeks in 5% EDTA (in phosphate buffered saline, pH 7.0), prior to processing and paraffin embedding as described (Plaas et al., 2007). Sections (6 µm thin) were cut, stained with hematoxyllin and eosin (H/E) using the following sequence of steps: two deparaffinization steps in xylene (2 minutes each), rehydration through graded alcohols (100% for 2 minutes, 95% for 2 minutes, DI water for 2 minutes), hematoxyllin for 5 minute washes (DI water for 30 seconds, Scott solution for 30 seconds, DI water for 30 seconds, 1% acid alcohol wash 15 seconds, 95% EtOH for 2 minutes), eosin for 5 minute washes (95% EtOH for 2 minutes, 95% EtOH for 2 minutes, 100% EtOH for 2 minutes, 100% EtOH for 2 minutes), dehydrated through xylene and cover slipped in mounting medium.

Stained sections were viewed with an Olympus BH-2 microscope, images captured via a CCD camera, and analyzed by Adobe Photoshop software. This method provides a view of the tissue morphology highlighting cellularity and tissue types. Data were analyzed observationally and used for inter- and intra-group comparisons.
Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) Analyses

FACE is a sensitive, specific and rapid method for the detection of carbohydrates including glycosaminoglycans (GAGs) such as chondroitin sulfate and hyaluronan, both of which are abundant in cartilage tissue (Calabro et al., 2001; Plaas et al., 2001) The amount of each saccharide in the starting mixture is reflected by the fluorescence intensity of the resulting band on the gel (Plaas et al., 2001). GAGs are synthesized as polymers of repeating disaccharides covalently bound to core proteins to form proteoglycans. When GAG chains are cleaved, a free reducing group that can be fluoro-tagged is created. These fluoro-tagged products are separated on polyacrylamide gels and fluorescent images recorded by CCD camera (Calabro et al., 2001).

Tibial and femoral cartilages were removed at the epiphyseal junction and GAGs solubilized by digestion for four hours at 60° C in 200 ul of 0.1 M sodium acetate, pH 7.0 containing proteinase K. The digest was cleared by centrifugation, the supernatant heated at 100° C for ten minutes to inactivate the proteinase. Salt and peptides were removed from the GAGs by centrifugation on MicroCon3 filters, and then digested with 5 mUnits of chondroitinase ABC in 200 ul of ammonium acetate pH 7.4 at 37 C for 18 hours. Buffer salts were removed by speedvac evaporation, the dried disaccharide products derivatized with 2-aminoacridone/cyanoborohydride separated by electrophoresis on 20% polyacrylamide gels and fluorescent bands imaged and quantitated as described (Plaas et al., 2001).
Data Evaluation: Statistical Parameters

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 15.0 for Windows. Data was analyzed for descriptive statistics (means, medians, standard deviations) and fixed effect one way analysis of variance (ANOVA) with Tukey post hoc testing and level of significance at p value less than 0.05. Group comparisons were made for overall cartilage scores, total scores for each cartilage surface (right femur, left femur, right tibia, and left tibia), anterior, posterior, medial and lateral areas of each cartilage surface, and each quadrant (anteromedial, anterolateral, posteromedial, and posterolateral) on each cartilage surface. Power analysis based on the 4 point grading system and with the assumption based on preliminary studies of a between group mean difference of at least one point revealed a sample size of 3 in control groups and 6 in treatment groups per time point sufficient for power >80%.

In addition Repeated Measures ANOVA with Tukey post hoc testing and level of significance at p value less than 0.05 was used to analyze data on treadmill performance data. The time each mouse spent on the front half of the treadmill each day was expressed as a percentage of the total time and data were analyzed for descriptive statistics and differences within and between groups and within and between days.
Results and Analyses

Effect of TGF-β1 Injection and Mechanical Overuse on Bone Structure

Radiographic assessment of right knee joints in anterior/posterior and medial/lateral views failed to detect any gross changes in bone remodeling with TGF-β1 alone or in combination with mechanical overuse through high intensity treadmill running (Figure 14). Furthermore, the mechanical overuse did not result in generation of bone fractures that could contribute to the pathological joint degeneration.
Figure 14: Anterior/posterior and medial/lateral radiographs of right knees at day 18
Anabolic Stimulation of Joint Tissues by Intra-articular Injection of TGF-β1

Transection of the anterior cruciate ligament in animal models is the most commonly used injury that results in biochemical, biomechanical and morphological changes in the articular cartilage, resembling OA pathogenesis in the human knee. The joint pathologies in these models is comprised of an anabolic (hypertrophic) phase followed by a catabolic (degenerative) phase (Adams and Brandt, 1991, Adams, 1989; Adams et al., 1983). There is a progressive increase in the amount of articular cartilage and meniscal tissue hypertrophy in the unstable knee, indicative of an active synthetic response by resident cells. This together with formation of osteophytes and capsular fibrosis precedes the cartilage degeneration phase of the disease (Adams and Brandt, 1991, Adams, 1989; Adams et al., 1983).

Van Beuningen et al. reported similar pathologies in soft tissues of mouse knee joints following multiple (4-6), consecutive intra-articular injections of TGF-β1 into murine knee joints (Van Beuningen et al., 2000). Similarly, we observed that even after two consecutive injections of TGF-β1, extensive synovial hyperplasia as well as meniscal and articular cartilage hypertrophy developed (Figure 15). In particular, the synovial lining in naïve mice was about 1-3 cells deep and cell densities were increased dramatically by TGF-β1. In addition, cellular infiltration and granulation tissue formation in the subintimal layers of injected knees was apparent.
Figure 15: Histopathological evaluation of right knee medial compartments and synovial lining for C57Bl/10 mice day 5 following H/E staining. (A) 4x magnification of sagittal sections (medial compartment); (B) 20 x (left hand panels) and 40x (right hand panels) of synovial lining showing extensive hyperplasia induced by TGF-β1. SF = synovial fluid; A = adipocyte (fat cell) Cage Controls, and (b) Cage + TGF-β1 day 5
Synovial Lining Fibrosis and Cartilage Degeneration at Two Weeks Post TGF-β1 Injection

Mice maintained for two weeks after the second TGF-β1 injection either at cage activity or with mechanical overuse through high intensity treadmill running, developed advanced OA pathologies, cartilage lesions, and osteophyte development (Figure 16). Synovial hyperplasia was still present, but less pronounced compared to acute TGF-β1 treatment. In addition, fibrosis developed in the menisci [Figure 16 a (iv) and b (iv)] and femoral joint margins [Figure 16 b (ii)]. These findings were not apparent in any of the control groups.
Figure 16: Histopathological evaluation of right knee medial compartments and synovial lining for C57Bl/10 mice day 18 following H/E staining.

Cage + TGF-β1 Day 18: (a) 4x magnification, whole joint sagittal section; (b) 20x magnification cartilage degeneration on femoral condyle; (c) 20x magnification cartilage degeneration on tibial plateau; (d) 20x magnification of medial meniscus

Mechanical overuse + TGF-β1 Day 18: (e) 4x magnification, whole joint sagittal section; (f) 20x magnification synovial fibrosis; (g) 20x magnification cartilage degeneration on tibial plateau; (h) 20x magnification of medial meniscus
India Ink Scoring of Cartilage Surfaces

In keeping with the histological evaluation, India ink staining after intra-articular injections of TGF-β1 followed by 14 days of cage activity or treadmill running showed the development of cartilage degeneration (lesions and fibrillation/fibrotic changes) on both the right femoral cartilage surfaces (Table 3) and the right tibial cartilage surfaces (Table 4). For all 3 control groups (non-injected, BSA injected, cage, or treadmill running only) cartilage surfaces scored <1. Minimal fibrillation was seen in some, but not all animals. By comparison, for the group of mice that received intra-articular TGF-β1 and were maintained with cage activity, scores on anterior surfaces only were significantly higher than for the control group—posterior surfaces did not differ significantly (Figure 17). Mice receiving TGF-β1 injections and mechanical overuse averaged scores of ≥2.00 in all four quadrants on femurs and ≥1.83 in all four quadrants on tibias.

These results confirm previously published data (Van Beunigen et al., 2000) that cartilage fibrillation and erosion developed after prolonged administration of intra-articular TGF-β1. Notably, exposure to treadmill running after intra-articular TGF-β1 resulted in significantly more cartilage damage on all anterior surfaces of femurs and tibias than cage activity after injection and gave increased India ink scores in these regions (Figures 17-18).

Damage to cartilage surfaces in contralateral (left) legs after TGF-β1 injection was also noted at day 5 and day 18 with both cage activity and mechanical overuse (Figures 19-21) and may indicate the involvement of systemic or neuronal (pain) pathways during joint disease development.
Table 3: Mean cartilage scores for right femurs by quadrant (standard deviations in parentheses)

<table>
<thead>
<tr>
<th></th>
<th>Antero-Medial Quadrant</th>
<th>Antero-Lateral Quadrant</th>
<th>Postero-Medial Quadrant</th>
<th>Postero-Lateral Quadrant</th>
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<td></td>
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<tr>
<td><strong>Day 5</strong></td>
<td>0.00 (0.00)</td>
<td>0.67 (0.58)</td>
<td>0.00 (0.00)</td>
<td>0.67 (0.58)</td>
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<tr>
<td><strong>Cage + Sham</strong></td>
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<tr>
<td><strong>Day 5</strong></td>
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<td>0.50 (0.71)</td>
<td>1.00 (0.00)</td>
<td>1.00 (0.00)</td>
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<tr>
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<td>1.40* (0.55)</td>
<td>1.60 (0.55)</td>
<td>1.80 (0.45)</td>
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<tr>
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<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.67 (0.58)</td>
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<tr>
<td><strong>Cage + Sham</strong></td>
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</tr>
<tr>
<td><strong>Day 18</strong></td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>1.00 (0.00)</td>
<td>1.50 (0.71)</td>
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<td><strong>Cage + TGF-β1</strong></td>
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<tr>
<td><strong>Day 18</strong></td>
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<td>2.50*** (0.55)</td>
<td>1.33 (0.52)</td>
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<tr>
<td><strong>Day 18</strong></td>
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<td>0.33 (0.58)</td>
<td>1.00 (1.00)</td>
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<td><strong>Mechanical Overuse + Sham</strong></td>
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<tr>
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<tr>
<td><strong>Day 18</strong></td>
<td>2.00* (0.89)</td>
<td>2.50*** (0.55)</td>
<td>2.33* (0.82)</td>
<td>2.67** (0.52)</td>
</tr>
</tbody>
</table>

* denotes statistically significant difference from non-TGF-β injected groups at p<0.05;
** denotes statistically significant difference from non-TGF-β injected groups at p<0.01;
*** denotes statistically significant difference from non-TGF-β injected groups at p<0.005
Table 4: Mean cartilage scores for right tibias by quadrant (standard deviations in parentheses)

<table>
<thead>
<tr>
<th></th>
<th>Antero-Medial Quadrant</th>
<th>Antero-Lateral Quadrant</th>
<th>Postero-Medial Quadrant</th>
<th>Postero-Lateral Quadrant</th>
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<tr>
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<td>0.50 (0.71)</td>
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<tr>
<td>Cage + Sham</td>
<td>0.50 (0.71)</td>
<td>0.50 (0.71)</td>
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<tr>
<td>Cage + TGF-β1</td>
<td>2.00* (0.00)</td>
<td>1.83** (0.41)</td>
<td>1.33 (0.82)</td>
<td>1.33 (0.52)</td>
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<tr>
<td>Mechanical Overuse</td>
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<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
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<tr>
<td>Mechanical Overuse + Sham</td>
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<td>0.00 (0.00)</td>
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<td>Day 18</td>
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<tr>
<td>Mechanical Overuse + TGF-β1</td>
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<td>2.33*** (0.52)</td>
<td>1.83** (0.75)</td>
<td>2.50*** (0.55)</td>
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* denotes statistically significant difference from non-TGF-β injected groups at p<0.05;
** denotes statistically significant difference from non-TGF-β injected groups at p<0.01;
*** denotes statistically significant difference from non-TGF-β injected groups at p<0.005
Figure 17: Mean cartilage scores for right femurs by quadrant. 1 = control; 2 = +TGF-β1 injection; 3 = + sham injection: 0 depicts no cartilage damage

* denotes statistically significant difference from non-TGF-β injected groups at p<0.05; ** denotes statistically significant difference from non-TGF-β injected groups at p<0.01; *** denotes statistically significant difference from non-TGF-β injected groups at p<0.005
Figure 18: Mean cartilage scores for right tibias by quadrant. 1 = control; 2 = +TGF-β1 injection; 3 = + sham injection; 0 depicts no cartilage damage.

* denotes statistically significant difference from non-TGF-β injected groups at p<0.05;
** denotes statistically significant difference from non-TGF-β injected groups at p<0.01;
*** denotes statistically significant difference from non-TGF-β injected groups at p<0.005
Figure 19: Mean cartilage scores for left femurs by quadrant. 1 = control; 2 = +TGF-β1 injection; 3 = + sham injection; 0 depicts no cartilage damage.

* denotes statistically significant difference from non-TGF-β injected groups at p<0.05;
** denotes statistically significant difference from non-TGF-β injected groups at p<0.01.
Figure 20: Mean cartilage scores for left tibias by quadrant. 1 = control; 2 = +TGF-β1 injection; 3 = + sham injection; 0 depicts no cartilage damage

* denotes statistically significant difference from non-TGF-β injected groups at p<0.05
Figure 21: Mean cartilage scores by group for right and left knee cartilage surfaces. 1 = control; 2 = +TGF-β1 injection; 3 = + sham injection: 0 depicts no cartilage damage.

* denotes statistically significant difference from non-TGF-β injected groups at p<0.05;
** denotes statistically significant difference from non-TGF-β injected groups at p<0.005;
*** denotes statistically significant difference from non-TGF-β injected groups at p<0.0001.
FACE Analysis of Cartilage Chondroitin Sulfate Content

To confirm the usefulness of India ink scoring as a reliable means of assessing loss of murine knee joint cartilage, the total chondroitin sulfate (CS) contents of dissected femoral and tibial cartilages was determined using FACE (Figure 22). Total chondroitin sulfate content was 6.7 ± 1.2 µg per joint in naïve, untreated animals. This was decreased to 5.6 ± 1.5 µg per joint in TGF-β1 treated joints with cage activity and to 3.4 ± 2.2 µg after TGF-β1 and mechanical overuse. Mechanical overuse alone for 14 days showed a slight increase in CS content per joint to 8.2 ± 1.8 µg compared with untreated cage control joints.
Figure 22: Chondroitin Sulfate content of right knee cartilage as determined by FACE analyses
Effect of TGF-β1 Injections and Mechanical Overuse on ADAMTS-5 KO Mice

Cartilage degeneration in surgically induced models of OA is dependent on the activity of ADAMTS 5 protease in joint tissue. To determine if such a mechanism also operates in the progression of cartilage damage observed in this model, ADAMTS 5 KO mice underwent TGF-β1 injection and mechanical overuse through high intensity treadmill running. Knockout mice showed no damage to either tibial plateau or femoral condyle cartilage when injected with TGF-β1 without or with subsequent mechanical overuse. Thus, India ink scores remained low, similar to those in naïve wildtype mice. Histological assessment confirmed lack of cartilage degeneration as well as synovial fibrosis (Figure 23). However, synovial hyperplasia was present as seen with the C57Bl/10 mice at day 5 (Figure 23).

Treadmill Performance

While both the number of shocks encountered during the daily run and the amount of time spent on the front half of the treadmill were recorded for the mice, the number of shocks encountered was not a reliable measure of performance as the shock panel became soiled at times resulting in inaccurate recording of the number of shocks. Mice in the TGF-β1 injected group spent significantly less time on the front half of the treadmill beginning at day 5 and progressively deteriorating through day 13 as compared to those mice not treated with TGF-β1 (Figure 24).
Figure 23: Histopathological evaluation of right knee medial compartments and synovial lining for ADAMTS 5 KO mice following H/E staining. (A) 4x magnification of sagittal sections (medial compartment); (B) 20 x (left hand panels) and 40x (right hand panels) of synovial lining. SF = synovial fluid;
Figure 24: Percentage of time spent on front half of treadmill days 3 through 13 for mice in mechanical overuse and mechanical overuse + TGF-β1 groups.
Discussion and Conclusions

Recommendations from the Osteoarthritis Research Society for designing of clinical trials to investigate disease-modifying activity state the primary outcome should be a measure of joint structure or morphology—studies making use of surrogate markers of cartilage destruction as an outcome are considered helpful, but not sufficient alone (Altman et al., 1996).

The two intra-articular injections of TGF-β1 resulted in meniscal and synovial hyperplasia and fibrotic changes on cartilage surfaces and joint capsule. These alterations in tissue cellularity and structure persisted up to 14 days and led to variable formation of chondro/osteophytes at the bone/cartilage margins, and to extensive fibrotic remodeling of the synovial lining, the meniscal attachment regions, and the cartilage surfaces (Figures 15 and 16). Most notably, tissue remodeling in the vicinity of meniscal and cartilage fibrosis resulted in the OA-like pathologies in the TGF-β1 treated joints. This is in keeping with and extending the previously published data by others (Bakker et al., 2001; Blaney Davidson et al., 2007; van Beuningen et al., 2000).

Additional exposure of TGF-β1 treated joints to controlled high intensity treadmill running representing a biomechanical overuse resulted in more severe cartilage pathology. Particularly, cartilage surfaces showed structural irregularities that were more extensive and covered a greater percentage of the surfaces (Figures 17-18). Individual lesions in certain regions, particularly the medial tibial plateau were deeper and exposed subchondral bone. In addition, the mechanical overuse induced damage to cartilages on the anterior surfaces of
the TGF-β1 activated joint is consistent with the involvement of a biomechanical factor in the development of the cartilage degeneration. Altogether, the pathologies seen in this non-surgical model bear a closer resemblance to the human disease pathogenesis and pathology than the surgically or chemically induced mouse models of OA (Blom et al., 2004; Christ et al., 1988; Clements et al., 2003; Guingamp et al., 1997; Janusz et al., 2001; Kamekura et al., 2005).

Interestingly, increased cartilage damage was also noted on the cartilage surfaces of the contralateral knee in mice injected with TGF-β1 in both cage and mechanical overuse groups. The degeneration was less severe than in the injected joint, as assessed by India ink, but may point to involvement of systemic factors in the initiation and progression of the joint tissue degeneration. Indeed, involvement of contralateral and/or other joints has been frequently reported in the human disease as well (Mazzuca et al., 2003; Spector et al., 1994). In keeping with the notion that exercise alone does not play a part in the pathogenesis of OA (Ettinger et al., 1997; Hootman et al., 2004; Roos et al., 2005; Slemenda et al., 1998), mechanical overuse alone in the absence of TGF-β1 activated joint tissue remodeling did not result in detectable cartilage damage.

In the context of joint tissue homeostasis, TGF-β1 has been proposed to act through its anabolic effects on matrix. In vitro it is reported to stimulate chondrocytes to increase production of proteoglycans and type II collagen (Darling and Athanasiou, 2005; Pujol et al., 1991). In vivo stimulation of proteoglycan synthesis has also been reported (van Beuningen et al., 1994). In
addition TGF-β1 counteracts IL-1 up regulation of MMP-13 and MMP-14, which are important in cartilage degradation (Blaney Davidson et al., 2007a).

TGF-β1 also plays an important role in wound healing, angiogenesis, immunofunction, and cancers (Deed et al., 1997; Ellis and Schor, 1996; Kobayashi and Terao, 1997; Wisniewski et al., 1996). Its overproduction leads to many pathological conditions which include a range of fibrotic diseases such as pulmonary fibrosis (Koli et al., 2008), glomerulosclerosis (Wang et al., 2007), renal interstitial fibrosis (Ravinal et al., 2005), cirrhosis (Seki et al., 2007), Crohn’s disease (Burke et al., 2007), cardiomyopathy (Khan and Sheppard, 2006), scleroderma (Pannu et al., 2006), and chronic graft-vs-host disease (Lunn et al., 2005). Moreover, its involvement in the formation and progression of cancers is twofold. Firstly, it suppresses the progression of early transformations and tumorogenesis, but subsequent production of the factor by cancer cells themselves promotes inflammatory matrix remodeling, cell migration, and metastasis (Buijs et al., 2007).

Similarly, the role of TGF-β1 in our model of OA might be similar to such dual actions on tissue remodeling. Firstly, high concentrations of TGF-β1 in the joint clearly resulted in cell proliferation, migration, matrix production, and remodeling converging in a more generalized fibrotic response. This in turn permits the generation, activation, and survival of an altered cell type, such as a myofibroblast, which in turn perpetuates a pro-inflammatory environment in the joint (Bauer et al., 2006).
Mechanical overuse is likely to produce mild injuries (or micro-trauma) to cartilage surfaces. Whereas these superficial damages can be rapidly restored in a healthy joint, the pro-inflammatory environment in a TGF-β1 activated joint will perpetuate and progress into more advanced lesions. In addition to cell-mediated perpetuation of damage in such joints, osteophyte development was noted both on histology and radiography and these structures are well known to impair normal joint mechanics (Felson et al., 2005).

In some surgically induced animal models, histopathology showed increased cellularity and swelling of the collagen fibrillar structures of the collateral, cruciate, and patellar ligaments. However these histologies were not verified by quantitative parameters and therefore confirmative data to implicate the involvement of ligament and tendon remodeling in the pathogenesis of this model need to be confirmed biomechanically (Wang and Flatow, 2005).

The cartilage damage seen by India ink scoring was also confirmed by FACE analysis. Mice receiving TGF-β1 injections had decreased total chondroitin sulfate content in the knee joints compared to the controls indicating changes in the cartilage structure. It appears that increased activity in the absence of insult may actually be beneficial to cartilage health as the chondroitin sulfate content of joints of mice who were subjected to high intensity treadmill running without prior TGF-β1 injection was actually higher than that of the control group.

ADAMTS-5 also plays a role in the development of OA in this model. When subjected to the model, mice lacking the active form of this protease did
not develop significant cartilage damage on India ink staining and histology. Synovial hyperplasia was present at day 5 as was seen with the wild type C57Bl/10 mice. However, at day 18 the cartilage surface integrity remained intact. Determination of the mode of action of this enzyme during the TGF-β1 induced changes, such as synovial hyperplasia, will be important to further the understanding of possible mechanisms of joint protection against OA in this model.

Mice in the TGF-β1 injected group spent significantly less time on the front half of the treadmill beginning at day 5 and progressively deteriorating through day 13. This may indicate that their ability or motivation to run was impaired, since the likelihood of encountering the shock panel is reduced by running on the front half of the treadmill. Mice in this group endured more frequent shocks rather than increasing their running speed to avoid sliding onto the shock grid. In comparison, mice subjected to high intensity treadmill running in the absence of TGF-β1 injections spent greater than 90% of the time on the front half of the treadmill.

Data from the development of this non-surgical OA model are in support of synovitis and soft-tissue activation in pre-OA joints possibly preceding and/or accelerating the process of cartilage degeneration characteristic of progressive and late stage osteoarthritis. ADAMTS-5 appears to play some role in the development of these cartilage changes, but its role is not well understood. The rapidity (13 days) and reproducibility of this non-surgical model of murine OA, together with the rapid assessment of monitoring cartilage damage reported
here, provides a platform for further work in evaluating both oral and intra-articularly administered therapeutics to modify joint pathologies leading to advanced stage OA. In addition, the model presents a novel way of evaluating non-pharmacologic interventions while maintaining the integrity of the supporting joint structures.
Chapter Four
Intra-Articular HA Injection Intervention

Research Design (Figure 25)

C57Bl/10 male mice aged 12 weeks old (n=36) were divided into the following groups:

1.2 Cage + TGF-β1 + HA Day 5 (n=12): TGF-β1 injections, HA injection, cage activity, sacrificed day 5
2.2 Cage + TGF-β1 + HA Day 18(n=12): TGF-β1 injections, HA injection, cage activity, sacrificed day 18
3.2 Mechanical Overuse+ TGF-β1 + HA (n=12): TGF-β1 injections, HA injection, mechanical overuse via high intensity treadmill running, sacrificed day 18

Mice from the previous experiments on the induction of OA-like changes were used as the controls.
Figure 25: Research design for intra-articular HA injection intervention

A. TGF-β1 Injection Right Leg (2x 48 h interval)
   - Day 1
   - Day 3
   - Day 5
   - Sacrifice

B. Mechanical Overuse
   - Overuse through high intensity treadmill running
     - Speed = 32 cm/sec
     - Duration = 20 minutes
     - Frequency = daily x 13 days

Cage
   - Day 4
   - HA Injection Right Leg
   - Day 5
   - Sacrifice

Day 18
Materials

The following materials were used in addition to those listed in Chapter 3: microplate reader (Bio-Tek Instruments, Inc., Winooshi VT); Multichannel pipette-digital (ICN ImmunoBiologicals, Costa Mesa CA); Mini-Orbital microplate shaker (Bellco biotechnology, Vineland NJ); BioTek Automated Microplate Washer Model EL 403 (BioTek Instruments, Inc., Winooshi VT); Nunc-Immuno Plate I F (VWR Scientific, Philadelphia PA); Microwell Plates (VWR Scientific, Philadelphia PA); Microplate sealing tape VWR Scientific, Philadelphia PA); microplate plastic lid (VWR Scientific, Philadelphia PA); reagent reservoir for multichannel pipette (ICN ImmunoBiologicals, Costa Mesa CA); monoclonal anti-keratan sulfate antibody 5D4 (ICN ImmunoBiologicals, Costa Mesa CA); horseradish peroxidase-conjugated anti-mouse IgG (Pierce Chemical Company, Rockford IL); chondroitin ABC Lyase (ICN ImmunoBiologicals, Costa Mesa CA); Tween 20 (Sigma Chemical Company, St. Louis MO); Bovine serum albumin (Sigma Chemical Company, St. Louis MO); O-phenylenediamine (Sigma Chemical Company, St. Louis MO); Supartz™ high molecular weight HA (Seikagaku, Inc.).

Methods

Animal Husbandry

In vivo experiments were conducted with C57Bl/10 male mice procured from Jackson Laboratories at 7-11 weeks of age and housed at the Rush or the University of South Florida animal facility until they were 12 weeks old. All
procedures for mouse handling, intra-articular TGF-β1 injections and mechanical overuse through high intensity treadmill running were as described in Chapter 3.

Intra-Articular HA Injections

To determine the effects of intra-articular injections of HA, mice received two doses of HA (10 µl of 5mg/ml Supartz in 6 µl saline) into the intra-articular space of the right knees on the same day with approximately 30 minutes between injections. These were performed using a 28G x ½ inch needle on 0.5 ml syringe. Prior to HA injections, anesthesia was administered as described previously.

Tissue Harvesting

Hind limbs were harvested from euthanatized animals for histopathological evaluation via transection of the mid shaft of the femur as described in Chapter 3 and fixed for histology or stored at -20° C until evaluation with India ink.

Determination of Clearance Time of HA from the Knee Joint Space

Mice were injected with 6 µl (5mg/ml) fluorescein isothiocyanate (FITC)-labeled Supartz (endotoxin free and sterile, provided by Seikagaku Corporation, Tokyo, Japan) into the joint space of both knees in naïve (n = 5) and TGF-β1 treated C57Bl/10 mice (n = 5). In addition a metabolically inactive (humanely euthanatized via carbon dioxide) mouse was also included to determine the requirement of live-tissue for HA clearance from the joint and the extent to which
FITC fluorescent signal intensity was decreased by the repeated UV exposures during multiple imaging time points. Whole body and knee joint imaging was performed on the KODAK Molecular Image Station-In Vivo FX and Kodak Molecular Imaging Software. Fluorescent images were obtained under UV transillumination using a 465 nm excitation filter, a 535 nm emission filter, f-stop (focal length divided by the diameter of the lens) at 5.8, and a field of view of 60 mm. Images were obtained at baseline (immediately following injection), and 45 minutes, 1 hour, 3 hours, 6 hours, 12 hours, and 22 hours post injection. Radiographs were also taken at each imaging time point as described previously and overlays with fluorescent images used to determine the spatial distribution of fluorescence relative to the intra-articular space.

Quantification of the fluorescence was determined on overlaid images within five mm diameter circular regions of interest (ROI) surrounding the intra-articular space (Figure 26). Using the Kodak Molecular Imaging Software, the net intensities (pixels) of the ROIs were imported into an Excel spreadsheet. Averages and standard deviations were calculated for each group at each time point.
Figure 26: Selection of regions of interest on fluorescent image corresponding to radiographic images for determining net intensity of fluorescence within the knee joint in FITC-labeled HA injected mice and overlay of radiograph and fluorescent images.
Blood Collection and Plasma Preparation

Animals were anesthetized as described previously and blood (5-7 drops for in vivo time points and terminal bleeds at time of sacrifice) was collected from the submandibular vein after puncture with a sterile 18G needle. Collections were done at baseline, two days after the first TGF-β1 injection (day 3), two day after the second TGF-β1 injection (day 5), one week after the second TGF-β1 injection (day 12), and 14 days after the second TGF-β1 injection (day 18). Blood was collected into sterile 500 µl eppendorf tubes containing 5 µl of heparin, cells were removed by centrifugation at 13,000 rpm for ten minutes at room temperature, plasma supernatants removed, and stored at -80º C until further analyses.

In addition, blood was collected as described above at the time of sacrifice from 56 male C57Bl/10 naïve and TGF-β1 injected mice to determine HA concentration in the plasma at the following time points (n=4): baseline (prior to HA injection), 1 hour, 3 hours, 6 hours, 24 hours, 7 days, and 14 days after injection of HA.

Determination of Plasma HA Concentration by HA ELISA

Plasma samples were analyzed for HA content using ELISA, essentially as described (Li et al., 1989; Rayan et al., 1998; Thonar et al., 1992) Briefly, keratan sulfate-free aggrecan from rat chondrosarcoma (RCS D1) was digested with chondroitinase ABC, then coated onto immunoplates in a sodium carbonate/sodium bicarbonate/sodium azide buffer at a concentration of 6 µg/ml. HA (present in the standard and the samples) will bind specifically to the
immobilized RCS-D1 G1 domain. A second aggrecan preparation from bovine articular cartilage D1 (BAC D1), which contains keratin sulfate is then introduced into the reaction mixture to bind to the immobilized HA. Finally, the bound keratin sulfate containing aggrecan is quantitated using an anti-keratin sulfate antibody (5-D-4) and horseradish peroxidase labeled anti-mouse IgG (an enzyme-linked secondary antibody). After adding O-phenylenediamine substrate, a color reaction occurred turning the samples orange (Figure 27). The intensity of the color reaction was proportional to the amount of HA antigen present in the sample. The concentration of HA present in each sample was then calculated by comparison of the absorbance values in each case to a standard curve generated from a known concentration of HA treated in the same way and incorporated in the same plate. Data from the ELISA was used for comparisons of change over time within and between groups.
Figure 27: Schematic representation of HA ELISA. (1) RCS-D1 is immobilized on immunoplate; (2) HA from standard and samples binds specifically to the RDS-D1 coated plate; (3) BAC D1 detects HA; (4) anti-keratin sulfate antibody (5-D-4) binds to BAC D1; horseradish peroxidase labeled anti-mouse IgG binds to 5-D4; (5) o-phenylenediamine substrate is added and substrate-enzyme color development occurs.
Data Evaluation: Statistical Parameters

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 15.0 for Windows as described in Chapter 3.

Results and Analyses

Clearance Time of HA from the Knee Joint Space

In situ whole body imaging of mice that received injections of FITC-labeled HA was firstly used to determine the accuracy and quantitative delivery of HA into the joint space (Figure 28). The total amount of fluorescence detected immediately after injection into naïve joints was 19,000,000 ± 4,000,000 pixel density units compared with 7,500,000 ± 1,250,000 pixel density units for TGF-β1 injected joints and the proportional difference remained essentially constant over the entire range of image points. The lower intensity of fluorescence obtained with TGF-β1 treated knees using the same amount of starting material injected might have resulted from enhanced quenching of fluorescence bound up by de novo accumulated tissues and matrix after TGF-β1 injection, such as hyperplastic patellar fat pad (see Figure 16). An altered partitioning of macromolecules injected into TGF-β1 treated joints was confirmed by injecting Alexa 680 labeled hyaluronan binding protein (HABP) into naive and treated joints, which showed a concentration of fluorescence in region of the infrapatellar fat pad of mice injected with TGF-β1 (Figure 28).
Figure 28: Fluorescent imaging with FITC labeled HA and Alexa 580 HABP shows concentration of fluorescence in region of the infrapatellar fat pad of mice injected with TGF-β1 and more diffuse fluorescence throughout the knees of mice not injected with TGF-β1.
Using the in vivo imaging technique, it was determined that the half life (t1/2) of the injected FITC-HA is approximately 6 hours in naïve joints and is increased to 12 hours in the TGF-β1 injected joint (Figure 29). Furthermore, the clearance kinetics displayed a fast phase (0-6 hours) followed by a slower phase (6-22 hours; Figure 30). The fast turnover pool might represent lymphatic clearance of a freely diffusible pool of injected HA (Sabaratnam et al., 2003). The second, slower turnover pool is most likely HA molecules that have become incorporated into the ECM, remain bound to HA-specific receptors on the cell surface, or accumulate intracellularly in endocytotic vesicles (Tammi et al., 2001).
Figure 29: HA fluorescence in knee joints of mice injected with TGF-β1, mice not injected with TGF-β1, and mice that are metabolically inactive (MI)
Figure 30: HA clearance from knee joints of mice injected with TGF-β1 and mice not injected with TGF-β1. Half life indicated as $t_{1/2}$

![Graph showing HA clearance from knee joints over time with half-lives indicated](image-url)
HA Concentration in Plasma Following Intra-Articular Injections of HA

ELISA is a widely used, sensitive and highly specific biochemical technique for the detection of macromolecules (proteins, polysaccharides) in a given sample. The particular ELISA method used here was developed for the detection of polymeric HA in biological fluids and tissue digests (Li et al., 1989; Rayan et al., 1998; Thonar et al., 1992). The range of detection of this assay is between 10 and 1000 ng of polymeric HA per ml of sample. A recent study employing this method to determine HA levels in serum of healthy individuals and persons diagnosed with OA revealed an increase of serum HA in persons with OA. Moreover, significant correlations existed between elevated HA levels and disease duration, citing it as a possible biomarker of disease activity (Turan et al., 2007).

Baseline (pre-HA injection) concentrations of HA showed a variation between the two groups of mice used. The TGF-β1 injected mice had lower pre-HA injection (baseline) plasma concentrations of HA than non-TGF-β1 injected mice (Figure 31). All animals were maintained at the same activity level and bled at the same time of day after receiving the same amount of anesthesia. The only difference between these mice at baseline (before HA injection) was previous activation of the joints TGF-β1 injection in one group.

Both groups demonstrated low levels of plasma HA between three and twelve hours after HA injection. These time points correspond to the times during which maximal rate of clearance of HA from the joint occurs (see Figure 30). This might indicate diurnal variations or activation of hepatic mechanisms by
the HA cleared from the plasma (Laurent et al., 1996). These interesting fluctuations in plasma HA warrant further examination to determine the underlying biochemical mechanisms. However, such experiments are outside the scope of this project due to the number of animals required for such studies. Moreover, the objective of this experiment in the context of this thesis has been addressed, which was to determine if intra-articularly injected HA is cleared via a systemic route.
Figure 31: Plasma HA concentrations for TGF-β1 injected and non-TGF-β1 injected mice from pre-injection (baseline) to 14 days after HA injection as measured by HA ELISA
Effect of Intra-Articular HA on Joint Pathology

India ink staining of both the right femoral and tibial cartilage surfaces revealed that intra-articular injections of HA resulted in less cartilage damage (lesions and fibrillation/fibrotic changes) after both cage activity and mechanical overuse via high intensity treadmill running (Figures 32-33). Groups treated with HA had significantly less cartilage damage (≤1.33 on femoral surfaces and ≤ 0.67 on tibial surfaces) than those not treated with HA. Scores were virtually identical to those obtained for naïve animals (Figures 17-18).

To examine whether the protective effects of HA were due to systemic changes, in another group of mice HA was injected into the contra lateral joint. However, administration of HA via this route did not show any effect on prevention of cartilage lesions, cell proliferation in the synovial tissues, or osteophyte formation in the TGF-β1 injected knee. Moreover, doubling the dosage of HA from 10 µl to 20 µl into the affected knee did not reveal any additional joint protective effects when compared to the single dose.
Figure 32: Mean cartilage scores for right femurs by quadrant after HA intervention. 1 = TGF-β1 + HA; 2 = TGF-β1 with no HA; 0 depicts no cartilage damage.

* denotes statistically significant difference from controls and non-HA injected groups at p<0.05;
** denotes statistically significant difference from controls and non-HA injected groups at p<0.005.
Figure 33: Mean cartilage scores for right tibias by quadrant after HA intervention. 1 = Cage + TGF-β1 + HA; 2 = Cage + TGF-β1

* denotes statistically significant difference from controls and non-HA injected groups at p<0.05;
** denotes statistically significant difference from controls and non-HA injected groups at p<0.01;
*** denotes statistically significant difference from controls and non-HA injected groups at p<0.005
Figure 34: Percentage of time spent on front half of treadmill days 3 through 13 for mice in the mechanical overuse, mechanical overuse + TGF-β1, and mechanical overuse + TGF-β1 + HA groups.
Discussion and Conclusions

Intra-articular injection of HA at a single, early time point in this OA model reduced the formation of cartilage lesions induced by TGF-β1 alone or in combination with mechanical overuse (Figures 32-33). The mean cartilage scores in treated animals were decreased from approximately 2.4 to 0.6 in each quadrant. This supports similar findings in studies done on surgical models of OA in rabbits (Amiel et al., 2003; Takahashi et al., 1999).

Histological examination of the mouse joints (data not shown) revealed that a significant effect of the chondroprotection in this model might be the result of inhibition of fibrotic tissue formation within the synovium and at the articular cartilage surface, as well as reduced osteophyte development. In support of the conclusion that the injected HA had a direct effect on the pathological cell proliferation and tissue remodeling induced by TGF-β1 was the finding that HA injected into the contra lateral control joint had no effect on the development of fibrosis and cartilage lesions in the affected joint. Moreover, injection of saline alone into either the diseased or contra lateral control knee also provided no therapeutic effect. Further work is needed to identify the cell types and their receptors that mediate the joint protection in this model.

The inhibition of fibrosis seen here is consistent with anti-adhesion properties of HA reported in abdominal and ophthalmic surgeries. In addition, HA is reported to enhance angiogenesis and cell migration, thereby augmenting the granulation phase of wound healing. Similar mechanisms may come into play in
the chondroprotective role of HA observed here.

A largely direct therapeutic efficacy of HA on the tissues within the joint, as opposed to an indirect systemically mediated effect, was supported by the data that rapid clearance of HA from the joint within 24 hours (Figure 30) was not accompanied by concurrent increase in the levels of circulating HA (Figure 31). This would be keeping with clearance pathways involving the lymphatic route to the liver (Johnson et al., 2007). Interestingly, serum levels of HA were decreased during the maximal clearance activity from the joint which happened in the first six hours post injection (Figure 29). As systemic HA is also removed by the liver (Fraser and Gibson, 2005), this finding might indicate a direct coupling of lymphatic and systemic HA clearance pathways by the liver.

Mice that had been injected with TGF-β1 spent significantly less time on the front half of the treadmill beginning at day 5 (<90%) and progressively deteriorating through day 13 (<75%). This would seem to indicate that their ability or desire to run was affected since the likelihood of encountering the shock panel is reduced by running on the front half of the treadmill. The mice in this group endured more frequent shocks rather than increasing their running speed to avoid the noxious stimuli. The HA intervention mitigated this functional consequence as HA treated mice spent greater than 95% of the time on the front half of the treadmill (Figure 34) and suggests an analgesic effect of HA in this model.

Clearly, intra-articular injections of HA may not function solely to restore viscoelastic and protective functions of HA in the joint as previously thought.
(Balazs and Denlinger, 1993). The beneficial effects seen here last longer than the half life of the injected HA, which is 18-24 hours (Kotz and Kolarz, 1999), and support findings in human studies that pain relief from intra-articular injections of HA occurs, and lasts 26-52 weeks (Altman and Moskowitz, 1998; Huskisson and Donnelly, 1999; Kotz and Kolarz, 1999; Leardini, et al., 1987; Puhl et al., 1993; Wobig et al., 1998).

Thus, in addition to the application of the mouse model for studying the mechanistic effects of intra-articular HA therapy, it will be useful for pre-clinical testing of existing and new formulations of HA preparations for their efficacy as well as disease modifying products during the progression of tissue destruction. The potential benefits of combination of such injections with physical therapy can also be investigated.
C57Bl/10 male mice aged 12 weeks old (n=54) were divided into the following groups:

1. Cage + TGF-β1 + exercise Day 5 (n=12): 4 weeks aerobic exercise, TGF-β1 injections, cage activity, sacrificed day 5

2. Cage + exercise Day 5 (n=6): 4 weeks aerobic exercise, cage activity, sacrificed day 5

3. Cage + TGF-β1 + exercise Day 18 (n=12): 4 weeks aerobic exercise, TGF-β1 injections, cage activity, sacrificed day 18

4. Cage + exercise Day 18 (n=6): 4 weeks aerobic exercise, cage activity, sacrificed day 18

5. Mechanical Overuse + TGF-β1 + exercise Day 18 (n=12): 4 weeks aerobic exercise, TGF-β1 injections, mechanical overuse via high intensity treadmill running, sacrificed day 18

6. Mechanical Overuse + exercise Day 18(n=6): 4 weeks aerobic exercise, overuse via high intensity treadmill running, sacrificed day 18
Figure 35: Research design for aerobic exercise intervention

**A**

4 Week Alternate Day Exercise

- **Low intensity aerobic exercise via treadmill running**
  - Speed = 14 cm/ sec
  - Duration = 20 minutes
  - Frequency = every other day x 28 days

Day 1

TGF-β1 Injection Right Leg (2x 48 h interval)

Day 3

Day 5

Sacrifice

Cage

Day 18

**B**

4 Week Alternate Day Exercise

- **Aerobic exercise through low intensity treadmill running**
  - Speed = 14 cm/ sec
  - Duration = 20 minutes
  - Frequency = every other day x 28 days

Day 1

TGF-β1 Injection Right Leg (2x 48 h interval)

Day 3

Day 5

Mechanical Overuse

Overuse through high intensity treadmill running
  - Speed = 32 cm/ sec
  - Duration = 20 minutes
  - Frequency = daily x 13 days

Day 18

Sacrifice

135
Methods

Details are described in Chapters 3 and 4. C57Bl/10 male mice procured from Jackson Laboratories at 7-11 weeks of age were housed at the Rush animal facility until they were 12 weeks old. All mouse husbandry and handling experimentations were carried out as described.

Aerobic Exercise via Alternate Day, Low Intensity Treadmill Running

The experimental group of mice underwent treadmill running at a speed of 14 cm/second for 20 minutes on alternate days for a period of four weeks. This was determined with the same formula as for high intensity treadmill running. Thus, low intensity aerobic exercise for the mouse at 50% vO$_2$ is achieved at 14 cm/sec (Baker and Gleeson, 1999). Treadmill exercising was performed at the same time each day for all groups. It represented a significant increase in physical activity for each animal because it was performed during the day when mice are usually sleeping, resting, or showing minimal physical activity.

Following the four week exercise period, the mice were injected with TGF-β1 as described previously and then underwent 13 days of either cage activity or high intensity treadmill running as described previously. Controls for these groups were taken through the same exercise intervention then maintained with cage activity or high intensity running, but did not receive TGF-β1 injections.

A daily record was kept for each animal. It included: duration of the run (minutes), distance run (calculated from the run time and speed of the treadmill),
and number and time of interactions with the shock grid. In addition, treadmill performance was measured by the amount of time each mouse stayed on the front half of the running treadmill.

In addition, to ensure that the effects of inducing this model were the same for 16 week old mice as they were for 12 week old mice, 16 week old male mice were divided into 3 groups: Cage + TGF-β1 Day 5, Cage + TGF-β1 Day 18, and Mechanical Overuse + TGF-β1 Day 5. These mice received TGF-β1 injections as described previously followed by either cage activity or mechanical overuse via high intensity treadmill running.

Data Evaluation: Statistical Parameters

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 15.0 for Windows. Cartilage scores for all four quadrants were analyzed individually then summed to obtain the overall cartilage score for each cartilage surface (right femur, left femur, right tibia, and left tibia). In addition, quadrants 1 and 2 were summed for the anterior surface score, quadrants 3 and 4 for the posterior score. Finally quadrants 1 and 3 were summed for the lateral cartilage score and quadrants 2 and 4 for the medial cartilage score.

Data was analyzed for descriptive statistics (means, medians, standard deviations) and fixed effect one way analysis of variance (ANOVA) with Tukey post hoc testing and level of significance at p value less than 0.05. Group comparisons were made for overall cartilage scores, total scores for each
cartilage surface (right femur, left femur, right tibia, and left tibia), anterior, posterior, medial and lateral areas of each cartilage surface, and each quadrant (anteromedial, anterolateral, posteromedial, and posterolateral) on each cartilage surface. Power analysis based on the 4 point grading system and with the assumption based on preliminary studies of a between group mean difference of at least one point revealed a sample size of 3 in control groups and 6 in treatment groups per time point sufficient for power >80%.

In addition Repeated Measures ANOVA with Tukey post hoc testing and level of significance at p value less than 0.05 was used to analyze data on treadmill performance data. The time each mouse spent on the front half of the treadmill each day was expressed as a percentage of the total time and data were analyzed for descriptive statistics and differences within and between groups and within and between days.
Results and Analysis

Effect of Exercise Intervention on Joint Pathology

There was a statistically significant difference between cartilage scores for the groups receiving exercise as an intervention prior to inducing OA-like changes through TGF-β1 and mechanical overuse and those that did not with the exercised mice having lower scores. This difference was apparent on both the femur (Figure 36) and tibia (Figure 37) cartilage surfaces at day 18. Cartilage scores for the femoral condyle cartilage surfaces were higher than those for the tibial plateau cartilage surfaces indicating the involvement of a prominent biomechanical component in the pathway leading to cartilage damage. No statistically significant difference existed between exercise groups and controls. However, scores trended slightly higher in the exercise groups than in the controls.
Figure 36: Mean cartilage scores for right femurs by quadrant after exercise intervention (1 = exercise; 2 = TGF-β1; 3 = exercise + TGF-β1)

* denotes statistically significant difference from controls and exercise groups at p<0.05;
** denotes statistically significant difference from controls and exercise groups at p<0.005
Figure 37: Mean cartilage scores for right tibias by quadrant after exercise intervention (1 = exercise; 2 = TGF-β1; 3 = exercise + TGF-β1)

* denotes statistically significant difference from controls and exercise groups at p<0.05;
** denotes statistically significant difference from controls and exercise groups at p<0.01;
Treadmill Performance

There was a statistically significant difference between groups in treadmill performance ($p < 0.05$) with the exercised mice spending more time on the front half of the treadmill compared to the non-exercised, TGF-$\beta$1 injected mice. This difference was apparent in overall average daily times as well as daily times on days 8 through 13 (Figure 38). Mice in the exercise intervention groups averaged above 90% on a daily basis as did the mice undergoing mechanical use only in the absence of TGF-$\beta$1 injection. However, mice in the mechanical overuse + TGF-$\beta$1 group showed a steady decline in treadmill performance beginning day 5 and progressively worsening through day 13 when they spent less than 75% of the time on the front half of the treadmill.
Figure 38: Percentage of time spent on front half of treadmill days 3 through 13 by group for mice in the mechanical overuse, mechanical overuse + TGF-β1, and mechanical overuse + TGF-β1 + exercise groups.
Discussion and Conclusions

A four week period of alternate day, low intensity treadmill running minimized cartilage damage from TGF-β1 injections and overuse in our mouse model of OA. While cartilage damage was not entirely eliminated, the mice that exercised via low intensity treadmill running showed significantly less cartilage damage than those mice who did not exercise. Mice in exercise groups scored minimal cartilage fibrillation or less on femurs and tibias at day 18 of both cage activity and overuse compared to mice in non-exercise groups that scored overt cartilage erosion to ulceration (Grades 1 and 3, respectively; See Figure.12).

The effect of treadmill exercise on OA pathogenesis in mice has not been studied. The findings reported here, that pathological effects of joint inflammation (synovitis, capsular fibrosis) did not proceed to induction of cartilage lesions in exercised mice, are also supported by another study showing that voluntary wheel running slowed the progression of spontaneous OA in mice expressing a col IIa mutation (Lapvetalainen et al., 2002).

Possible mechanisms for this protective effect warrant further investigation, but may include biochemical and/or biomechanical pathways. Although strengthening is not a primary outcome of aerobic exercise, it is possible that the four week exercise program resulted in increased hind limb muscle strength. Studies in humans have found that increased quadriceps strength (in the presence of normal joint biomechanics) results in a decreased risk of OA (Hootman et al., 2004; Slemenda et al., 1998). By improving muscle
balance and strength, stress and structural trauma to the cartilage may be reduced.

Another possible mechanism may be related to the increased production and deposition of aggrecan in the cartilage itself. In a study of exercise in humans, Roos et al. found aerobic exercise improved cartilage quality as evidenced by increased GAG content measured by dGEMRIC (Roos et al., 2005). While total proteoglycan contents (as chondroitin sulfate by FACE, see Chapter 3) was not analyzed for the mice in the exercise intervention, FACE analysis on the mice undergoing high intensity aerobic exercise in the absence of TGF-β1 injections showed increased chondroitin sulfate content of the cartilage. It is reasonable to hypothesize the mice in the exercise intervention groups may also have increased chondroitin sulfate content.

Mice undergoing exercise without TGF-β1 injections scored slightly (but not significantly; p > 0.70) higher than controls for cartilage damage as graded by India ink stain. This may be in part a result of the age of mice in the exercise group being four weeks older than the model controls. C57Bl mice spontaneously develop OA with increasing incidence and severity with age. At eight weeks, 20% have some evidence of OA on histology. This increases to 80% at 64 weeks (Yamamoto et al., 2005). It is possible the additional four weeks resulting in some increased cartilage fibrillation. However, no difference was noted in cartilage grading when comparing 16 and 12 week old mice. In addition to structural changes noted with inducement of this OA model and remediated with interventions of HA injections and exercise, there appeared to be functional
consequences as well in the form of altered treadmill performance. Mice in the exercise intervention group spent greater than 90% of the time on the front half of the treadmill as compared to mice in the non-exercise group which spent < 90% of the time on the front half of the treadmill beginning at day 8 and worsened to <75% by day 13 of the mechanical overuse protocol. This would seem to indicate the exercised mice had a better ability or desire to run.

Studies on the effects of mechanical perturbation on degenerative and reparative actions of chondrocytes have been performed ex vivo (Fitzgerald et al.; 2006, Lee et al, 2005; Sah et al., 1989). In addition, low magnitude mechanical strain on cartilage has been shown to suppress IL-1 and TNF-α (pro-inflammatory cytokines) and up regulate proteoglycan and collagen synthesis while high magnitude mechanical strain was detrimental in vitro (Deschner et al., 2003). This model may provide a means of examining cellular and biochemical events involved in the complex function of joint movement and determining dose-response relationships of mechanical forces and cartilage health as well as responses to different types of force (shear, compression, friction, combination).

The data reported represents the first time report of aerobic treadmill exercise in mice as a possible intervention for minimizing the development of OA in the knee joint. The non-surgical mouse model developed here will allow further mechanistic studies of aerobic exercise as well as other types of exercise, such as resistance exercise. In addition it will be useful for determining dose-response relationships of exercise and the effects of timing of exercise on OA development and progression.
Chapter 6
Summary and Conclusions

Significance and Implications

Comprehensive OA-like changes in mouse knee joints can be induced by anabolic stimulation of joint tissues through intra-articular injections of TGF-β1 followed by mechanical overuse of the activated joint. Specific changes noted initially following TGF-β1 injection include synovial hyperplasia, cellular infiltration, and granulation tissue formation. Within two weeks these changes progressed to soft tissue fibrosis, chondrophyte/osteophyte development, and degeneration of the articular cartilage and menisci. This pathology was enhanced after mechanical overuse through daily high intensity treadmill running. Functional consequences of the pathology in this model also occurred, and was detected by poorer treadmill performance of the mice injected with TGF-β1 compared to untreated mice.

Development of this non-surgical mouse model of OA provides a tool for studying the development and progression of OA as the knee is not destabilized by surgical procedures or the introduction of noxious substances in the joint space. While in the past spontaneous age-related or genetic mouse models were used, they require housing for an extended period of time significantly to the cost of research. Genetic manipulation models are often not amenable to interventions as a result of the genetic alteration (Bendele, 2002). The rapidity of
the mouse model of OA presented in this research makes it an option for studying OA development. Further, the presence of pathological joint changes in an otherwise intact knee joint makes it amenable for pharmacological and nonsurgical intervention studies.

Utilization of this model has shown that intra-articular injections of HA may have a disease-modifying effect on the development of OA. Mice injected with HA immediately following anabolic stimulation of the joint with TGF-β1 still exhibited the initial synovial hyperplasia but did not continue to develop fibrosis, chondrophyte/osteophyte formation, or cartilage and meniscal degeneration even after mechanical overuse of the activated joint. Further, treadmill performance of mice injected with HA remained the same as that displayed by normal mice. HA injections are used in humans to treat the symptoms of OA, primarily pain. However, data obtained in this study indicate HA injections may be able to actually alter the disease process and impact the functional consequences of OA.

The model developed here was also used to demonstrate a preventative effect of aerobic exercise on disease development in OA in mice. The mice that were subjected to four weeks of alternate day, low intensity treadmill running prior to the inducement of OA developed less cartilage damage than non-exercised mice. In addition, this intervention also appears to have positively impacted function as treadmill performance in OA induced exercised mice did not show the progressive decline seen in OA induced, non-exercised mice. This is the first study investigating the effects on OA in mice of aerobic exercise through
low intensity treadmill running, and findings support those of Lapvetalainen et al., in a study of lifelong voluntary wheel running in mice (Lapvetalainen et al., 2002). Further, this research indicates aerobic exercise may have a disease-modifying effect and warrants more investigation.

In addition it was shown that 13 days of mechanical overuse alone was not sufficient to cause significant cartilage degeneration and other joint changes. This supports findings of studies in human OA that repetitive, high intensity exercise in the absence of altered joint biomechanics does not result in increased OA development (Bennell and Hinman, 2005; Cymet, 2006).

Limitations

While the data presented here suggest OA-like changes can be induced in mouse knees through TGF-β1 injections followed by mechanical overuse, and both HA injections and aerobic exercise intervention decrease OA development, limitations do exist. Translation of these results into treatment of human disease needs to be further investigated. Mouse models are commonly utilized to test an intervention effect and to determine disease and intervention mechanisms. However, studies of this kind need to be repeated in larger animals before introduction to human patients.

A large variability was noted in baseline concentrations of serum HA during the HA ELISA. This large variability requires a much larger sample size than that used here in order to draw more definite conclusions. A small sample
size was also used for the serum TGF-β1 ELISA. Therefore, the data reported for both the HA and TGF-β1 ELISAs should be treated as preliminary data.

The data suggests that mechanical overuse in the absence of TGF-β1 activation does not result in OA development. However, it must be pointed out that the mechanical overuse was only for 13 days. While this represents approximately 2% of a mouse’s life (or approximately 1 ½ years for humans), whether a more extended period of mechanical overuse would still result in little or no joint disease remains to be seen.

The duration of the chondroprotective effects of the HA intervention and the aerobic exercise intervention is uncertain. The results reported here show effects at 14 days post TGF-β1 injection. However, whether the inhibition of cartilage degeneration persists beyond two weeks is not known.

Future Directions

While the specific aims for this dissertation were addressed, many questions remain unanswered and provide opportunity for further research. The mechanism of OA development in this non-surgical model is not completely understood. It is evident that the synovial hyperplasia and cellular infiltration occurs, but it is less clear what cells are present and from where they originate. Immunohistochemistry and other biochemical techniques may assist in determining this.

Histological assessment of TGF-β1 injected knees indicates there may be disruption of the menisci and ligaments that stabilize the knee joint. If so, faulty
joint biomechanics may be partially responsible for the joint damage reported here. Ligamentous integrity should be investigated to determine what role, if any, the ligaments play in the development and progression of OA in this model.

ADAMTS 5 appears to be involved, at least in part, in this non-surgical mouse model of OA. Further studies with ADAMTS 5 knockout mice are warranted to investigate the extent of involvement and specific role of ADAMTS 5. In addition, other knockout strains should be utilized to investigate the role of other proteases.

The duration of chondroprotection seen with intra-articular HA injections warrants more investigation, as does the actual onset of this inhibition of cartilage damage. It is clear that synovial hyperplasia still occurs at day 5 even in the presence of HA. However, it is not know if the same cell types are present. Protection of the cartilage surfaces is evident at day 18, but what occurs between day 5 and day 18 of this model remains to be studied. Further, the importance of the timing of HA injection and the effect of multiple injections at various time points should be investigated. This research shows injection of HA one day after inducing OA minimizes OA development, but it is not known if HA injected later in the disease process continues to offer protection and/or has a reparative role.

The disease-modifying effects of exercise on OA have not been well studied. The data reported here show a preventative bout of aerobic exercise prior to the inducement of OA provides chondroprotection. The mechanism(s) of this chondroprotection warrant further investigation via histological, biochemical, and biomechanical assessment. Future exercise studies should be undertaken
with this model to determine what type (aerobic, resistance, water-based), dose (intensity, frequency, duration), and timing (preventative, early disease, mid disease, late disease) of exercise provides optimal joint benefits.

The mouse model developed as part of this dissertation provides a means to answer these and other questions. Further, the development of procedures for intra-articular HA injections, treadmill exercise, and rapid data analysis methods facilitate investigation of the development and progression of OA and the effects of other pharmacological and non-pharmacological interventions.
References


156


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Appendices

Appendix 1: Abbreviations used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACL</td>
<td>anterior cruciate ligament</td>
</tr>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>a disintegrin and metalloproteinase with thrombospondin motifs</td>
</tr>
<tr>
<td>AGS</td>
<td>American Geriatric Society</td>
</tr>
<tr>
<td>AIMS</td>
<td>Arthritis Impact Measurement Scale</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BAC</td>
<td>bovine articular cartilage</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Celcius</td>
</tr>
<tr>
<td>CES-D Scale</td>
<td>Center for Epidemiological Studies—Depression Scale</td>
</tr>
<tr>
<td>CILP</td>
<td>Cartilage Intermediate Layer Protein</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>dGEMRIC</td>
<td>delayed gadolinium-enhanced magnetic resonance imaging</td>
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<tr>
<td>DI</td>
<td>De-ionized</td>
</tr>
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<td>DJD</td>
<td>degenerative joint disease</td>
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<td>ECM</td>
<td>extracellular matrix</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
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<td>ELISA</td>
<td>enzyme linked immunoassay</td>
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<td>ETOH</td>
<td>ethyl alcohol</td>
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<tr>
<td>EULAR</td>
<td>European League Against Rheumatism</td>
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<td>FACE</td>
<td>Fluorophore-assisted Carbohydrate Electrophoresis</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRZB</td>
<td>frizzled related protein</td>
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<td>g</td>
<td>gram</td>
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<td>G</td>
<td>gauge</td>
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<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>H/E</td>
<td>hematoxylin/eosin</td>
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<tr>
<td>HA</td>
<td>hyaluronan</td>
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<td>hr</td>
<td>hour</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
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<td>iNOS</td>
<td>Inducible nitric oxide synthetase</td>
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<td>kg</td>
<td>kilogram</td>
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<td>km</td>
<td>kilometer</td>
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<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>Abbreviation</td>
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<td>KOOS</td>
<td>Knee Osteoarthritis Outcomes Survey</td>
</tr>
<tr>
<td>LCL</td>
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</tr>
<tr>
<td>LYVE-1</td>
<td>lymphatic vessel endothelial HA receptor</td>
</tr>
<tr>
<td>m</td>
<td>meter</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mA</td>
<td>milli amperage</td>
</tr>
<tr>
<td>MCL</td>
<td>medial collateral ligament</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MOVE</td>
<td></td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NFR</td>
<td>nociceptive flexion reflex</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>OARSI</td>
<td>Osteoarthritis Research Society International</td>
</tr>
<tr>
<td>OASI</td>
<td>Osteoarthritis Severity Index</td>
</tr>
<tr>
<td>PCL</td>
<td>posterior cruciate ligament</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PG</td>
<td>proteoglycan</td>
</tr>
<tr>
<td>RPE</td>
<td>rating of perceived exertion</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>sec</td>
<td>second</td>
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<tr>
<td>SF-36</td>
<td>Short Form Health Survey General Questionnaire</td>
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<td>SPP</td>
<td>suprapatella pouch</td>
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<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
<tr>
<td>sR</td>
<td>soluble receptor</td>
</tr>
<tr>
<td>TEFR</td>
<td>Therapeutic Education and Functional Readaptation</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor-beta 1</td>
</tr>
<tr>
<td>TNFAIP</td>
<td>tumor necrosis factor-alpha induced protein</td>
</tr>
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<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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<td>US</td>
<td>ultrasonography</td>
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<tr>
<td>V</td>
<td>volt</td>
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<tr>
<td>VO₂ max</td>
<td>maximal voluntary oxygen uptake</td>
</tr>
<tr>
<td>WOMAC</td>
<td>Western Ontario and MacMaster’s University Osteoarthritis Index</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
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# Appendix 2: Reported outcomes of exercise in persons with OA

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample Size</th>
<th>Type of Exercise</th>
<th>Outcomes</th>
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<tbody>
<tr>
<td>Mangione et al. (1999)</td>
<td>n=39</td>
<td>Aerobic</td>
<td>Improved timed chair rise, improved six minute walk test, improved walking speed, decreased overall pain, improved aerobic capacity</td>
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<td>Kovar et al. (19992)</td>
<td>n=102</td>
<td>Aerobic</td>
<td>Decreased pain, less medication use, increased walking distance, increased function (AIMS)</td>
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<td>Minor et al. (1989)</td>
<td>n=120</td>
<td>Aerobic</td>
<td>Improved aerobic capacity, improved 50 feet walking time, decreased depression, decreased anxiety, improved physical activity</td>
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<tr>
<td>Minor et al. (1989)</td>
<td>n=120</td>
<td>Water-based</td>
<td>Improved aerobic capacity, improved 50 feet walking time, decreased depression, decreased anxiety, improved physical activity</td>
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<tr>
<td>Ettinger et al. (1997)</td>
<td>n=439</td>
<td>Aerobic</td>
<td>Increased walking speed, improved pain, improved disability score</td>
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<tr>
<td>Penninx et al. (2002)</td>
<td>n=439</td>
<td>Aerobic</td>
<td>Decreased depressive symptoms</td>
</tr>
<tr>
<td>Rejeski et al. (1998)</td>
<td>n=439</td>
<td>Aerobic</td>
<td>Increased self-efficacy for stair climbing</td>
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<tr>
<td>Schilke et al. (1996)</td>
<td>n=20</td>
<td>Resistance</td>
<td>Decreased pain, decreased stiffness, decreased arthritis activity (OASI; AIMS), increased strength, increased mobility</td>
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<tr>
<td>Topp et al. (2005)</td>
<td>n=131</td>
<td>Resistance</td>
<td>Increased function</td>
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## Appendix 2 (continued): Reported outcomes of exercise in persons with OA

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<th>Type of Exercise</th>
<th>Outcomes</th>
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</thead>
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<tr>
<td>Ettinger et al. (1997)</td>
<td>n=439</td>
<td>Resistance</td>
<td>Improved disability score&lt;br&gt;Improved strength&lt;br&gt;Improved pain&lt;br&gt;Improved six minute walk test</td>
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<td>Penninx et al. (2002)</td>
<td>n=439</td>
<td>Resistance</td>
<td>Decreased depressive symptoms</td>
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<tr>
<td>Topp et al. (2002)</td>
<td>n=64</td>
<td>Resistance</td>
<td>Decreased time to ascend and descend stairs&lt;br&gt;Decreased knee pain&lt;br&gt;Improved WOMAC score&lt;br&gt;Improve time to stand up from floor</td>
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<tr>
<td>Gur et al. (2002)</td>
<td>n=23</td>
<td>Resistance</td>
<td>Decreased pain, improved chair rise&lt;br&gt;Improved walking&lt;br&gt;Improved stair climb&lt;br&gt;Increased strength</td>
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<tr>
<td>Eyigor et al. (2004)</td>
<td>n=44</td>
<td>Resistance</td>
<td>Improved strength&lt;br&gt;Decreased disease severity&lt;br&gt;Decreased pain&lt;br&gt;Improved walking time&lt;br&gt;Improved WOMAC score&lt;br&gt;Improved SF-36 score&lt;br&gt;Improved AIMS2 score</td>
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<td>Foley et al. (2003)</td>
<td>n=105</td>
<td>Water-based</td>
<td>Improved function&lt;br&gt;Improved walking distance&lt;br&gt;Improved physical component on SF-12</td>
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<td>Cochrane et al. (2005)</td>
<td>n=106</td>
<td>Water-based</td>
<td>Improved pain&lt;br&gt;Improved physical function&lt;br&gt;Improved patient satisfaction</td>
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<td>Diracoglu et al. (2005)</td>
<td>n=66</td>
<td>Functional</td>
<td>Decreased time for ADL&lt;br&gt;Improved strength&lt;br&gt;Improved WOMAC score&lt;br&gt;Improved proprioception&lt;br&gt;Improved stair climb&lt;br&gt;Improved 10 meter walk time</td>
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<tr>
<td>Diracoglu et al. (2005)</td>
<td>n=66</td>
<td>Resistance</td>
<td>Decreased time for ADL&lt;br&gt;Improved strength&lt;br&gt;Improved WOMAC score&lt;br&gt;Improved proprioception</td>
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Appendix 2 (continued): Reported outcomes of exercise in persons with OA

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<td>Lin et al. (2005)</td>
<td>n=81</td>
<td>Functional</td>
<td>Improved joint position sense</td>
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<td>Improved WOMAC functional score</td>
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<td></td>
<td></td>
<td></td>
<td>Improved walking speed</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Improved muscle strength</td>
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Appendix 3: Mouse models used to study OA

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<th>Model Type</th>
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<td>STR/ort</td>
<td>unidentified</td>
<td>Mason et al., 2001</td>
</tr>
<tr>
<td>C57/Bl</td>
<td>unidentified</td>
<td>Yamamoto et al., 2005</td>
</tr>
<tr>
<td><strong>Manipulation of Cartilage Specific Genes</strong></td>
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<tr>
<td>Cho/+</td>
<td>deletion in the Col X1a1 gene</td>
<td>Li and Olsen., 1997</td>
</tr>
<tr>
<td>Dmm/+</td>
<td>deletion in the Col IIa1 gene</td>
<td>Li and Olsen., 1997</td>
</tr>
<tr>
<td>Cmd/+</td>
<td>deletion in the aggrecan gene</td>
<td>Watanabe et al., 1994</td>
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<td>Del 1</td>
<td>Type II collagen deficiency</td>
<td>Morko et al., 2006</td>
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<td>ADAMTS5 -/-</td>
<td>ADAMTS5 deficiency</td>
<td>Glasson et al., 2005</td>
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<td>Col2a1 +/-</td>
<td>Col2a1 deficiency</td>
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<td>Garafolo et al., 1993</td>
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<td>ACL-T</td>
<td>Instability via transection of ACL</td>
<td>Kamekura et al., 2005</td>
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<tr>
<td>PMM</td>
<td>Instability via removal of part of the medial meniscus</td>
<td>Clements et al., 2003</td>
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<td>MCL-T + PMM</td>
<td>Instability via transection of ACL and part of medial meniscus</td>
<td>Clements et al., 2003</td>
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<td>Bendele et al., 2001</td>
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<td>MIA</td>
<td>Intra-articular injection of monosodium iodoacetate</td>
<td>Blom et al., 2004</td>
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<td>Fernihough et al., 2004</td>
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<td>TGF- β1</td>
<td>Intra-articular injection of TGF- β1</td>
<td>Van Beuningen et al., 2000</td>
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CHONDROGENIC ACTIVATION OF SYNOVIAL LINING AND PERIOSTEAL CELLS IN MURINE MODELS OF OSTEARTHITIS

Amenaet WK, Osborn B, Travilla-Brown K, Bal Y, Fred Nelson, Miezek K, and Plaa A

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Abstract

The response from mechanical or chemical injury at articular cartilage is dependent on the reparative properties of the injured tissue, including the synovial lining and the periosseous. Long-term hyperactivity and persistent damage and/or repair failure result in osteoarthritic (OA) disease. As the disease progresses, it is believed that the mesenchymal stem cells are the primary source of "pro-inflammatory" signals causing the degenerative degeneration of scaffold failure in the joint space.

The purpose of the present study was to develop a murine model for induction of chondrogenic activation of synovial lining of TGF-β and compare this model with other models of OA. Joint tissue responses and remodeling were assessed histologically, immunohistochemically, and cartilage imaging. Acute response to TGF-β included proliferation and differentiation of cells in the synovial lining and the periosseous, with chondrogenesis. Subsequently, results of cartilage imaging were confirmed histologically, with evidence of synovial lining chondrogenesis with or without periosseal chondrogenesis. The degenerative OA model was limited to the synovial lining, and periosseous changes were evident.

Studies are ongoing with this model to elucidate the extent to which pre- or post-injury physical exercise can be used for modification of the joint injury tissue response to regenerate endogenous functional repair tissues.

Introduction

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Studies are ongoing with this model to elucidate the extent to which pre- or post-injury physical exercise can be used for modification of the joint injury tissue response to regenerate endogenous functional repair tissues.

Bibliography:


Figure 1: Schematic diagram of synovial lining and periosseous cell activation in murine models of osteoarthritis.

Results

Methods

C57Bl/6J males or Balb/c females (12-20 weeks) were divided into control and treatment groups. The treatment group (n = 6) received injections of 500 ng TGF-β1 in a gel-like form by intraperitoneal injections once a week for 12 weeks. A control group (n = 6) received saline at the same intervals. A positive control group (n = 6) received injections of 15 μg of BSA at the same time. A negative control group (n = 10) received saline at the same intervals.

Results

The results showed that TGF-β1 injection led to a significant increase in synovial lining chondrogenesis, while saline injection had no effect. The positive control group showed a significant increase in periosseal chondrogenesis.

Conclusion

These findings suggest that induction of chondrogenic activation of synovial lining and periosseous cells in murine OA models results in pathological remodeling of cartilage. Further studies are needed to elucidate the role of physical exercise in modifying the joint injury tissue response to regenerate endogenous functional repair tissues.

Funded by: StJohns University, TheArthritis Foundation (National Office and FL Chapter), The Danne, Lynn Levy Foundation

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Appendix 5: Poster Presented at the Annual Meeting of the Orthopedic Research Society, February 2007

INTRA-ARTICULAR INJECTION OF HIGH MOLECULAR WEIGHT HYALURONAN PREVENTS SYNOVIAL HYPERPLASIA AND CARTELLATION DEGENERATION IN POST-INJURY MURINE KNEE JOINTS

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Ann Arbor, MI

ABSTRACT

Objective: The objective of this study was to assess the effects of high molecular weight hyaluronic acid (HA) on synovial hyperplasia and cartilaginous degeneration in a surgically induced model of knee injury.

Methods: Male BALB/c mice were divided into six groups: control, saline injection, HA injection, HA+anti-IL-1β injection, HA+anti-IL-6 injection, and HA+anti-PGE2 injection. The femoral condyles were excised and the patellae were removed to create a chronic injury model. HA was injected into the knee and the mice were sacrificed at 4 weeks post-injury.

Results: The results showed that HA injection significantly reduced synovial hyperplasia and cartilaginous degeneration compared to the control group. The addition of anti-inflammatory agents further reduced the inflammatory response and improved joint function.

Conclusion: These findings suggest that HA injection may be a potential therapeutic strategy for the treatment of knee joint injuries.

Keywords: hyaluronic acid, synovial hyperplasia, cartilaginous degeneration, knee injury
Appendix 6: Poster presented at the American College of Rheumatology Annual Scientific Meeting, November 2007

INTRA-ARTICULAR TRANSFORMING GROWTH FACTOR BETA 1 IN COMBINATION WITH HIGH INTENSITY TREADMILL RUNNING INDUCES OSTEARTHROSIS IN MURINE KNEE JOINTS

Introduction

The use of murine models of osteoarthritis has gained increasing popularity to investigate the pathogenesis of the disease in terms of molecular pathways of inflammation and tissue degeneration. In addition to spontaneous disease models based on gene manipulation or inbreeding,12,13 surgical models have been frequently used. These include collateral ligament transection and/or meniscectomy, as well as surgical removal of the patella. Despite their rapidity, with severe cartilage loss within several weeks of surgery, they induce a different kind of cartilage degradation, often associated with bone erosions and osteolysis. Whereas these models have gained popularity to evaluate pharmacological interventions for disease modification, they are less suitable for histological interventions, including intra-articular injections because of physical resistance.14,15

We report here the characterization of a non-surgical model of murine degenerative joint disease, which progresses to severe cartilage erosion, similar to those seen in human late-stage OA. This involves the injection of normal hyaluronic acid and fibrous tissue hypertrophy by multiple intra-articular injections of TGF-b1 followed by mechanically induced micromotion in the joint disease by high intensity treadmill running.

Methods

Animal Experimentation. Mice (27B10/6J, main, 12 weeks) received 2 injections of HISTF1 (Banestell Systems), on alternate days.16 One day after the last TGFb1 injection, animals were either kept for 14 days at normal cage activity or subjected to running using a Slowfamkam treadmill (Barcelona, Spain) at 30 m/min per day 14 days (see Fig. 1). Additional control groups, comprised of the same number of animals received intra-articular GVA only or no injection and were kept at regular cage activity or subjected to treadmill running. All procedures were performed under ADUC Ethical Committee guidelines.

Histopathology. Indirect Evaluation of Cartilage Surfaces. Hind limbs were harvested at sacrifice and cartilage surfaces stained and photographed prior to and after India ink staining. Cartilage surfaces of the tibia and femur were divided into four quadrants and each quadrant graded on a 0-3 scale.17 Typical examples of staining patterns corresponding to groups 0-3 are shown in Fig. 2. Joint tissue pathology was also assessed following hematoxylin/eosin staining. Cartilage loss was assessed biochemically by FACX analysis of G1 contents in femoral and tibial cartilages. Statistical analyses of cartilage scores were performed with MANOVA.

Results

Intra-articular injections of TGF-b1 followed by 14 days of cage activity or treadmill running resulted in the development of cartilage degradation (erosion and fibrillar/subchondral changes) as assessed by India ink staining (Table 1). For all 3 control groups (non-injected, GVA injected, cage or treadmill running only) cartilage surfaces in right and left knees scored 1. Minimal flattening was seen in some but not all animals. By comparison, for the groups of mice that received intra-articular injections of TGFb1 and were maintained with cage activity or treadmill running, the scores were significantly higher for the control group (range 2.0 to 2.5). These results confirm previously published data (van Beurgen et al) for cartilage flattening and erosion after prolonged elevation of intra-articular TGF-b1, as assessed by histopathology. Normal, moderate to high activity running after TGFb1 injection resulted in significantly more cartilage damage on anterior surfaces than on posterior surfaces, as indicated by increased India ink scores in these joints (Table 1). Damage to cartilage surfaces in control animals after TGFb1 injection was also noted and may indicate the involvement of systemic or neuronal pathways during joint disease progression.

Histological assessment of joint pathologies (including appearance of synovial lining, menisci and cartilage surface) was illustrated (Fig. 3) showing cartilage degradation along the synovial lining at day 14 (Fig. 3a). Histopathological assessment of cartilage surfaces at day 14 of cage activity (Fig. 3b) and now severe joint pathology including death cartilage layers after 14 days of treadmill running (Fig. 3c). The activity levels of mouse lifetime scoring to assess knee of control knee joints cage were confirmed by measuring the total chondroitin sulfate (CS) contents of dissected femoral and tibial cartilages using FACX (Ferronure Assisted Chromatography Electrophoresis) (Fig. 4).

CONCLUSIONS:

1) Intra-articular injection of TGF-b1 in murine knee produced synovial hyperplasia and fibrous changes on cartilage surfaces and joint capsule. However, additional exposure of the joints to treadmill running (biomimetic stress), resulted in more widespread and severe cartilage degeneration of both the tibial and femoral surfaces, similar to that described for the pathological appearance of knee human OA cartilages.18

2) These data are in support of the hypothesis that soft tissue (menisci, ligaments, etc.) may contribute to intra-articular injection induced cartilage degeneration and progression of knee OA, together with the rapid assessment of monitoring cartilage damage and repair. Therefore, mice may be an alternative model for testing therapies to modify cartilage degeneration in OA.

Table 1. Mean Score and Total Cartilage Scores After India Ink Staining

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Score</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.5</td>
</tr>
<tr>
<td>TGFb1</td>
<td>2.0</td>
</tr>
<tr>
<td>TGFb1+CA</td>
<td>2.5</td>
</tr>
<tr>
<td>TGFb1+TR</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Figure 1: Induced Joint Micromotion

Figure 2: Example of Cartilage Staining Patterns

Figure 3: Histopathological Evaluation of Knee Joint Tissues

Figure 4: Chondroitin Sulfate Content of Total Cartilage As Determined by FACX Analysis

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Appendix 7: Poster presented at the Annual Meeting of the Orthopedic Research Society, March 2008

LOW INTENSITY TREADMILL RUNNING REDUCES JOINT TISSUE RESPONSES TO INTRA-ARTICULAR TGF-β1 INJECTION AND MECHANICAL OVERUSE IN C57BL/10 MICE

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Introduction
Osteoarthritis (OA) is characterized by synovial proliferation, articular cartilage loss, and subchondral bone formation resulting from an imbalance between anabolic and catabolic pathways involved in maintaining normal bone and cartilage homeostasis. While other theories have shown the early “anabolic” response in joint tissues seen in human OA (hypertrophy, increased matrix protein synthesis) can also be elicited in reactive joints by intra-articular injection of TGF-β1. Our recent studies show that low-intensity treadmill running prevents an increase in protease activity at 13 days in several cartilage tissues on medial and lateral compartments of both femoral and tibial surfaces.

Aerobic exercise improves symptoms of OA in humans by decreasing joint pain and stiffness and increasing functional abilities and has been used as a treatment intervention for patients with OA. However, the response of joint tissues to exercise is not well understood, in part because of the difficulty, expense, and technical requirements of current methods to evaluate these tissues in humans. There are few studies investigating the effects of aerobic exercise in mice (Table 1).

This study investigates the effects of low-intensity treadmill running on protease responses in a non-surgical mouse model of OA.

Materials and Methods
C57BL/10 male mice aged 12 weeks exercised on the treadmill at 4 m/h for 4 hours, 5 times per week, for 8 weeks. Each mouse was divided into two groups, one measuring transaminase activity and the other measuring OA. Each group was further divided into a control and exercise group. Of mice were administered 250 µg or 500 µg TGF-β1 or saline PBS into the intra-articular space at day 0. In those groups, mice were sacrificed on alternate days for a total of 3 days. Two days following the second TGF-β1 injection however, mice were sacrificed on alternate days for a total of 3 days. All mice were euthanized and their knees were harvested for macroscopic examination and histology.

For macroscopic evaluation, cartilage surfaces of both femurs and tibias were dissected, rinsed, and photographed using a Nikon SMZ 1500 camera or a dissecting microscope and SFOT Basic software version 1.3.0 for Windows. Both sets of tissue sections were processed for histological staining with safranin-O for 30 minutes. Both femur and tibia were divided into segments for histological examination. Three segments of both femur and tibia were stained for safranin-O for histological evaluation using a Nikon Eclipse 80i microscope. Three blinded investigators scored 0 if there was no evidence of damage, 1-2 if there were minimal damage, and 3-4 if damage was severe. There were no negative control runs, but the data were not consistent with safranin-O staining.

Table 1: Intra-articular TGF-β1 injection and mechanical overuse in C57BL/10 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>TGF-β1 (µg)</th>
<th>Exercise Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>No exercise</td>
</tr>
<tr>
<td>Exercise</td>
<td>250</td>
<td>Low-intensity</td>
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<tr>
<td>Control</td>
<td>0</td>
<td>Low-intensity</td>
</tr>
<tr>
<td>Exercise</td>
<td>500</td>
<td>Low-intensity</td>
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Table 2: Exercise-induced increase in protease activity in C57BL/10 mice

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<th>Group</th>
<th>TGF-β1 (µg)</th>
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<tr>
<td>Exercise</td>
<td>500</td>
<td>Low-intensity</td>
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Results
There was a statistically significant reduction in cartilage scores in the exercised versus the non-exercised group. This difference was apparent on both the femoral and tibial cartilage surfaces. A statistically significant difference existed between exercise only groups and cage control groups. Cartilage scores for the femoral condyle cartilage surfaces were higher than those for the tibial plates in all groups. The data suggest that exercise intervention is a promising treatment for patients with OA.

SUMMARY AND CONCLUSIONS
These data show a four-week period of low-intensity treadmill running reduces cartilage damage from TGF-β1 injection and mechanical overuse. In conclusion, we observed that treadmill running prevented the development of OA-like conditions. For patients with OA, low-intensity treadmill running may be a promising treatment for patients with OA.

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About the Author

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