The Role of Catabolin in Experimental Osteoarthritis

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ABSTRACT

The pathogenesis of osteoarthritis (OA) is complex, but likely involves destruction of articular cartilage by endogenous enzymes (Dingle 1979). Factors controlling this are not well understood. Catabolin, a 21,000 molecular weight peptide structurally and functionally related to interleukin-1, stimulates living but not killed chondrocytes in vitro to degrade their matrix (Fell and Jubb 1977, Saklatvala et al. 1983), suggesting it is not itself a degradative enzyme but functions as a control factor. The work in this thesis investigated the possible role of catabolin in the pathogenesis of OA by measuring catabolin production by cultures of synovium excised from the canine anterior cruciate ligament transection model of OA. Normal canine synovium in culture was shown to produce a factor which can stimulate the release of glycosaminoglycans from living canine articular cartilage in culture. The total amount of catabolin produced by cultures of synovium from experimentally induced OA synovium is statistically significantly greater (p<0.05) than that produced by normal synovium. When calculated per gram of synovium, there was no statistically significant difference. This suggests that a possible role for catabolin in the pathogenesis of OA might be related to the degree of synovial hypertrophy.
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<tr>
<td>ACL</td>
<td>Anterior cruciate ligament</td>
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<tr>
<td>BNS</td>
<td>Bovine nasal septum</td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
<td></td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
<td></td>
</tr>
<tr>
<td>1-9DMB</td>
<td>1-9 Dimethylmethylene blue</td>
<td></td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
<td></td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
<td></td>
</tr>
<tr>
<td>IFBS</td>
<td>Heat inactivated fetal bovine serum</td>
<td></td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulphate</td>
<td></td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolts</td>
<td></td>
</tr>
<tr>
<td>MEM</td>
<td>Eagle's minimal essential medium</td>
<td></td>
</tr>
<tr>
<td>mA</td>
<td>Milliamperes</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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Chapter 1
INTRODUCTION
The purpose of this thesis is to explore the interaction of synovium and articular cartilage in osteoarthritis (OA). A recently described synovial protein, called catabolin (Dingle et al., 1979) stimulates chondrocytes to degrade their extracellular matrix. Since cartilage matrix degradation is a prominent feature of OA, catabolin has been suggested as a control factor in articular cartilage metabolism in OA. In the introduction to this thesis, I will define osteoarthritis, present brief relevant discussions of the epidemiology of OA, discuss the cartilage-synovium interactions in natural and experimentally induced osteoarthritic joints and finally, present an experimental approach to address these questions.

1.1 Definition

Osteoarthritis is a disease of unknown etiology affecting central and peripheral diarthrodial joints with pathological features which include erosion of cartilage, subchondral sclerosis, juxta-articular cysts and remodelling of the bone ends with formation of marginal osteophytes. These pathological features are reflected in the radiographic findings with loss of joint space, subchondral sclerosis, juxta-articular cysts and osteophytosis. The disorder is characterized clinically by pain, deformity and dysfunction of the joint. OA can be classified into primary and secondary forms. The primary or idiopathic form can involve peripheral and central joints with either single or multiple joint involvement. Within the primary form there are variant subsets including erosive inflammatory OA, generalized OA and diffuse idiopathic skeletal hyperostosis. The secondary form can be further subdivided and related to trauma, systemic metabolic diseases (e.g., ochronosis), endocrine disorders (e.g.,
acromegaly), calcium deposition diseases (e.g., calcium pyrophosphate) dihydrate, neuropathic disorders (e.g., Charcot joints), bone dysplasias (e.g., multiple epiphyseal dysplasia) and other joint disorders (e.g., avascular necrosis and rheumatoid arthritis). The articular and peri-articular changes characteristic of OA are likely the result of a common pathological pathway from a number of underlying disorders. The clinical spectrum is broad ranging from asymptomatic patients with incidental radiographic findings to patients with crippling generalized disease. Synonyms of osteoarthritis include arthrosis, osteoarthrosis, degenerative joint disease and degenerative arthritis.

1.2 Epidemiology

Osteoarthritis occurs in all mammalian species and in all races of man. 80% of all people over the age of 55 have radiographic but not necessarily clinical evidence of osteoarthritis (Kellgren, J.H. et al., 1958). Epidemiologic studies have shown a direct correlation between OA and aging with a rapid increase after 50 years of age (Lawrence et al., 1966). British Columbia Medical Services Commission statistics for the period April 1, 1980 to March 31, 1981 indicate the use of 36,383 hospital bed days at a cost of $8.04 million for which OA was the major responsible diagnosis. These figures do not reflect the outpatient costs, physician visits, drug costs, time loss from work and disability payments for patients with OA. The costs of pain and suffering with this disorder are inestimable.

1.3 The Normal Synovial Joint

An understanding of the normal diarthrodial joint is prerequisite to
the study of the pathological joint. Diarthrodial joints provide mobility for locomotion and positioning the limb in space. In the sixth week of embryogenesis diarthrodial joints are formed from cavities within the mesenchymal limb buds. The bones are formed from a cartilage premodel by a process called endochondral ossification where the cartilage is infiltrated by nutrient vessels forming primary and secondary centers of ossification. Between the centers of ossification is the physis which allows for longitudinal growth, while the ossification of the cartilage adjacent to the joint shapes the articular surface and leaves the ends of the bone capped with hyaline articular cartilage. Hyaline cartilage is a unique substance which is avascular, aneural and ideally suited to withstand the repetitive compressive loads of normal day to day living. The cells of cartilage, called chondrocytes, secrete an extracellular matrix composed of a fibrillar type II collagen and polyanionic hydrophilic molecules called proteoglycans. The collagen fibrils are arranged in an arcade like architecture to resist the shear and compressive forces across the joint. The hydrophilic proteoglycans exert an osmotic swelling pressure which is resisted by the collagen network. This balance of forces gives articular cartilage unique viscoelastic biomechanical properties.

Articular cartilage can be divided into three strata: a superficial or tangential zone, an intermediate zone, and a basal zone. The superficial zone contains few cells which lie parallel to the surface, they are arranged in line with the collagenous arcade. The chondrocytes in this layer are small and flat with relatively few organelles and resemble fibroblasts. The intermediate zone contains active synthetic cells rich in rough endoplasmic reticulum, Golgi apparatus, glycogen and lipid droplet inclusions and small mitochondria. Morphologically these cells are more
rounded and projecting from the cell membrane are cytoplasmic processes. The chondrocytes of the basal zone are large and aggregated into clusters. There is a reduction in the quantity of synthetic organelles and an increase in the glycogen content indicating less active cells.

The proteoglycans in cartilage are formed from polysaccharides called glycosaminoglycans (GAG) covalently bound to a protein core. The GAGs are linear polymers of repeating sulphated disaccharide units of variable length. The two most abundant GAGs in articular cartilage are chondroitin sulphate (CS) formed from sulphated N-acetyl galactosamine-glucuronic acid disaccharide subunits and keratan sulphate (KS) formed from sulphated N-acetyl glucosamine-galactose disaccharide subunits. The average molecular weight of CS chains slowly declined from 21,000 in the first year of life to 16,400 in the 81–95 year-old group (Hjertquist and Wasteson 1972). KS, in contrast, undergoes a modest increase in length and number (Sweet et al., 1979). With aging, the protein and keratan sulphate contents of the proteoglycans gradually increase, whereas the content of chondroitin sulphate decreases (Simunek and Muir 1972). These observed changes can be explained if it is assumed the hyaluronic acid-binding region and the KS-binding region remain constant in size, whereas the CS-binding region becomes smaller with age (Inerot et al. 1978).

The protein core to which the GAGs are linked is asymmetric with a linear portion to which the GAGs are bound and a globular portion with no GAGs attached. The linear portion is approximately 400nM in length the distal 3/4 of which is rich in chondroitin sulphate while the proximal 1/4 is rich in keratan sulphate. The globular portion, which is devoid of GAG, can non-covalently bind to another GAG, hyaluronate, to form a
supramolecular complex of aggregated proteoglycan. Two small proteins (molecular weight: 47,000 and 44,000 daltons) have been extracted from hyaline cartilage. These proteins are important in stabilizing the non-covalent binding of proteoglycans to hyaluronate and have been called link protein. Oligosaccharides, which compete with hyaluronate for binding sites on proteoglycans, can dissociate proteoglycan aggregates, but are ineffective in the presence of link protein (Hardingham, 1979). Individual proteoglycans have a molecular weight averaging 1-2 million daltons and in an aggregate form a molecular weight in the order of 50-100 million (Muir and Hardingham, 1975). The biological function of these macromolecules is not entirely clear although it appears that their enormous size and hydrophilic nature when entrapped in a collagen meshwork prevents extrusion of proteoglycans and gives cartilage viscoelastic properties.

Although cartilage appears as an inert tissue without cell replication, the chondrocytes are actively replacing their surrounding matrix. Using $^3$H labeled hydroxyproline and $^{35}$SO$_4$ labeled glycosaminoglycans, it appears the half life of $^{35}$SO$_4$ labeled glycosaminoglycans is ≈ 300 days and that collagen matrix turnover is one tenth of GAG turnover (Thompson 1981).

Articular cartilage has a limited capacity for repair because of its avascularity. The response of most tissues to injury can be divided into three phases: necrosis, inflammation, and repair. The second phase, inflammation is mediated almost entirely by a vascular response which is obviously not possible in articular cartilage. The chondrocytes can as a result of injury increase matrix synthesis and with chronic injury can replicate (Mankin and Lippiello 1970). While articular cartilage is unable
to repair superficial injury, when there is a full thickness injury down to subchondral bone vascular invasion occurs and healing of the defect may occur with production of biomechanically inferior fibrocartilage. Full thickness defects in immature rabbits under the influence of continuous passive motion can promote healing with hyaline articular cartilage (Salter et al. 1980).

While the articular portion of synovial joints are covered with hyaline articular cartilage, in some joints there are fibrocartilagenous menisci which assist in load transmission. These menisci are attached to the joint capsule and adjacent bones. Removal or injury of the menisci will lead to early degenerative changes. The non-articular portion of a joint is lined by a synovial membrane which is composed of two cell types called type A and B synoviocytes. The synovium, which is one to five cell layers thick, is not an epithelium as it lacks a basement membrane and yet it lines a cavity making it a unique structure in the body. The type A synoviocyte is a phagocytic cell and thought to be macrophage derived, while the type B synoviocyte is a secretory cell derived from mesenchyme. This lining produces synovial fluid which is a transudate of plasma modified by the addition of glycoproteins. Synovial fluid is important for nutrition to chondrocytes and joint lubrication.

Cartilage surface wear is related to the coefficient of friction which is modulated by joint lubrication. There are many theories for joint lubrication including: hydrodynamic, elastohydrodynamic, boundary, and mixed. These theories apply the principles of lubrication with surface films but do not take into account some unique properties of articular cartilage. Weeping lubrication, (McCutchen 1959) extrusion of fluid from cartilage into the synovial fluid has been suggested to provide a
hydrostatic pressure keeping the surfaces separated. Walker et al. (1968) suggested that under conditions of load that fluid would flow from the synovial fluid into pores in the surrounding cartilage thus increasing the concentration of hyaluronic acid and the viscosity of the synovial fluid. It is likely that a number of factors are important in joint lubrication. Motion is also important for nutrition of articular cartilage. It has been shown in a dog model that immobilizing a joint for six weeks leads to a decrease in protein synthesis and cartilage atrophy (Palmoski et al. 1979).

The synovial membrane is also capable of producing a number of neutral and acid proteolytic enzymes including cathepsins and metalloproteinases, which may be important in the regulation of the extracellular matrix and in the pathogenesis of disease (Dingle 1979).

1.4 The Osteoarthritic Joint

In experimentally induced osteoarthritis the earliest biochemical changes were found to be an increase in water content. With progression of the disease the water content again declines (McDevitt and Muir 1976). Muir feels the collagen network has loosened allowing the proteoglycans to act with increased hydrostatic pressure. Increased water content is also seen in early preparations of human OA (Mankin and Thrasher 1975). Proteoglycans were more easily extracted from the osteoarthritic than normal cartilage which suggested the proteoglycans were less tightly immobilized in the collagen network (McDevitt and Muir 1974). In early OA there is an increase proteoglycan synthesis (Mankin and Lippiello 1971). Experimentally induced OA resulted in a 30% reduction of GAG in the areas of cartilage that went on to develop OA lesions (McDevitt 1979). In both experimental OA (McDevitt and Muir 1974, 1976) and human OA
(Mankin and Lippiello 1971) there is a decrease in the relative proportion of KS to CS using the galactosamine-glucosamine molar ratio. The CS enriched proteoglycans in experimental OA probably arise from altered biosynthesis in the initial stages of the disease. These biochemical changes were evident prior to any histologic change. Mankin and Lippiello (1971) using human OA cartilage noted an increase in chondroitin 4-sulphate with a fall in chondroitin 6-sulphate and keratan sulphate. They suggested that the chondrocytes from OA cartilage synthesized proteoglycans which resembled immature cartilage.

Reports of a change to type 1 collagen in osteoarthritic cartilage matrix, (Ninni and Desmikh 1973) could not be substantiated (Eyre and Muir 1974). In contrast to the marked changes in the GAG, collagen content in OA and normal articular cartilage is the same (Mankin and Lippiello, 1970). It is thought that the fatigue properties of collagen fibers, under the influence of mechanical stress, change to allow an increase in water content. In late OA there is morphologic evidence of collagen fiber disruption (Freeman and Meachim 1979).

A large number of proteolytic enzymes are present in cartilage and thought responsible for degradation of the proteoglycan and collagen components (Gross and Lapiere, 1962). These enzymes are active at either a neutral or acidic pH. Articular cartilage contains cathepsin B,D and F which may be involved in matrix regulation but have no activity at or above pH 7. The synovium contains both neutral and acid proteolytic enzymes including collagenase and synovial fluid contains neutral proteinases. Also present are a number of enzyme inhibitors which modulate the activity of these enzymes (Sellers and Reynolds 1977). Dingle feels the initial site for matrix degradation is close to the
chondrocyte in a protected pericellular environment which permits a high local concentration of enzymes allowing the chondrocyte to regulate matrix degradation (Dingle 1978).

For the purpose of description, the pathological changes in OA can be divided into: early, intermediate, and late stages. The early stage is preclinical and therefore asymptomatic. The biochemical changes are those described above for the collagen and proteoglycans. Grossly, the cartilage becomes opaque and yellowish. Histologic examination may show a loss of superficial staining, surface irregularities and a slight hypercellularity. In the intermediate stage there is further cartilage surface change with fibrillation, some chondrocyte death, other areas with clumping of chondrocytes and more extensive loss of staining. The cartilage is thinner as a result of abrasive wear. These lesions are usually focal. The late stage of OA is characterized by full thickness cartilage loss, exposure of subchondral bone with sclerosis, marginal osteophytes and subchondral cysts. Marginal osteophytes are boney projections capped by cartilage along the margin of articular cartilage which are formed from endochondral ossification. The pathogenesis of subchondral cysts is unclear although they may represent the cytotoxic effect of synovial fluid on bone (Meachim 1975). Apart from the articular change, there is capsular fibrosis and flexion deformity.

The etiology of OA is complex. There are currently three major categories of theories: abnormal physical stress and resultant biomechanical failure of articular cartilage, increased subchondral stiffness, and biochemical injury to the extracellular matrix with failure of repair. The biomechanical failure may be as a result of abnormal joint mechanics for example hip dysplasia with resultant interfacial wear.
producing surface loss and structural defects. Repetitive stress with fatigue wear causes disruption of the collagen meshwork and loss of GAG. These changes make the cartilage more susceptible to further mechanical injury. The ability of cartilage to resist wear declines significantly with age (Kempson 1979) supporting the theory of biomaterial failure.

Normal subchondral bone plays a major role in the dissipation of joint reaction forces. With subchondral fracture and subsequent healing there is an increase in subchondral stiffness and with more load being absorbed by articular cartilage. Radin et al. (1978), using an animal model with repetitive impulsive loading producing increased subchondral stiffness, showed early biochemical changes in the articular cartilage.

Cellular factors and enzymes are thought to be important in the pathogenesis of OA. Neutral metalloproteinases have been demonstrated to diffuse rapidly through cartilage and cause proteoglycan degradation (Ehrlich 1981), although their role in the pathogenesis of OA remains controversial. The synovial membrane is capable of elaborating proteolytic enzymes but are immediately taken up by $\alpha_2$ macroglobulin in the normal joint. Mankin and Lippiello (1970) have shown accelerated protein synthesis by chondrocytes in human osteoarthritic cartilage supporting the chondrocyte's role in repair in early OA. While there is increased synthesis of protein, overall there is a net loss for which degradative enzymes may be responsible.

Currently there is interest in synovial factors elaborated by synovium. These factors can initiate cartilage breakdown and are thought by some (Dingle 1984) to play a role both in the etiology and pathogenesis of OA. The above theories are not mutually exclusive and in OA it is
probable that multiple factors are involved.

1.5 Cartilage-Synovium Interactions

Fell and Jubb (1977) studied the cartilage-synovial interactions with co-culture experiments. Live and dead porcine cartilage was cultured either in contact with or at a distance from porcine synovium. These experiments showed two effects; the first a direct presumably enzymatic effect and the second an indirect effect mediated through living chondrocytes. When cartilage was cultured at a distance from synovium only living chondrocytes degraded their matrix. Further work by Dingle et al. (1979) demonstrated the breakdown of cartilage matrix by porcine synovial culture medium was due to a protein which they have called 'catabolin'. Catabolin stimulated matrix degradation only in living, not in killed cartilage, suggesting its action was not enzymatic but in some way regulatory.

Later experiments (Saklatvala and Dingle 1980) showed that catabolin was a thermolabile protein with a molecular weight of approximately 20,000 and an pI of 4.6. Up to 90% of cartilage matrix can be degraded when stimulated with catabolin suggesting that catabolin stimulated chondrocytes to degrade their matrix by release of proteinases. Not only can catabolin stimulate matrix degradation, Dingle (1984) was also able to show an inhibition of proteoglycan synthesis in organ culture. He suggested that in vivo such inhibition of synthesis, coupled with local degradative stimulation could damage articular cartilage and make the cartilage more susceptible to mechanical injury.

Interleukin-1(IL-1) is 12,000-18,000 dalton protein macrophage derived mediator which may be functionally and chemically related to catabolin. Its biological activities include: lymphocyte activation,
stimulation of hepatic acute phase protein production, induction of neutrophilia, and pyrogenicity. Wood et al. (1983) were able to isolate small quantities of an IL-1 like factor from the synovial fluid of inflamed joints and suggested it might contribute to joint destruction. The mechanism of action of IL-1 has not been entirely elucidated although it is thought to stimulate arachidonic acid release and thereby synthesis of prostoglandin $E_2$ (Baracos 1983). IL-1's ability to stimulate chemotaxis of neutrophils may contribute to the neutrophil invasion into inflamed joints. Dingle (1984) feels catabolin is distinct from IL-1 because of different molecular weights and $\pi_l$, although he agrees no firm claim can be made until these two substances are purified to homogeneity.

Steinberg and Sledge(1979, 1983a, 1983b) using a cocultivation model with human rheumatoid synovium and bovine nasal septum cartilage, showed a 1.5-3.5 increase in proteoglycan breakdown. The addition of a pharmacological concentration of hydrocortisone significantly suppressed synovium induced cartilage breakdown. Saklatvala (1981) using 0.1 $\mu$g/ml of cortisol succinate found less than 30% inhibition of GAG release and felt the addition of cortisol succinate would improve the assay without inhibiting any active components. Herman, Appel and Hess (1984) and O'Byrne et. al (1984) have demonstrated the production of catabolin by rheumatoid synovium. Herman, Appel and Hess (1984) and O'Byrne et. al (1984) have also presented evidence to suggest production of catabolin by human osteoarthritic synovium. At the present time there is no in vivo experimental work to suggest that catabolin is active in OA.

1.6 Experimental Osteoarthritis

Osteoarthritis has a long asymptomatic phase with the earliest
clinically definable changes appearing late in the course of the disease. Therefore to study the early changes in OA, it is necessary to use an experimental model. The disease produced should be a reasonable representation of the human condition, be reproducible and predictable. Animal models allow for control tissue and temporal assessment of joint changes. Commonly used models include spontaneous disease in mice, hip dysplasia in the dog, meniscectomy in the rabbit and cruciate ligament section in the dog (reviewed by Adams and Billingham 1982). The cruciate ligament section is one of the best studied models (Paatsama, 1952, Marshall and Olsson, 1971, Nuki and Pond, 1973, McDevitt and Muir, 1976, Adams et al. 1983). Marshall and Olsson (1971) observed the long term morphological changes which occurred 7 to 23 months after surgical section of the anterior cruciate ligament. They found osteophytes along the posterior aspect of the medial tibial condyle, along the femoral condyle, in the intercondylar groove and on the inferior pole of the patella. These osteophytes continued to enlarge up to one year postoperatively. The menisci often had tears, especially the medial meniscus. Most investigators consistently found degenerative changes using this model (Nuki and Pond 1973, McDevitt and Muir 1976, Adams et al. 1983), however Marshall and Olsson found degenerative changes in only 4 of the 10 operated dogs. From day four onwards ultrastructural changes were evident with separation of the collagen fibers (Stockwell et al. 1980). Synovial proliferation was evident one week postoperatively. The biochemical changes in the dog model have been presented above (McDevitt and Muir 1976). Six weeks post ligament section the cartilage was softer and thicker (McDevitt et al. 1977). There were deep clefts evident on the weight bearing portion of the femoral condyle by seven weeks.

The advantages of the canine cruciate section model of OA are: that
the joint is large enough to allow correlation of gross, histological and biochemical changes, the changes are a reasonable representation of the disease as it occurs in humans, the exact time of onset is known which allows for temporal assessment of disease severity, nonoperated contalateral and sham operated joints act as controls, and physical activity and environmental factors can be controlled. The synovium is easily dissected from the capsule of the joint and provides enough tissue for culture. Thus it is a good model to study cartilage synovium interactions.

1.7 Aims of Thesis

As discussed above, it has been postulated that catabolin is involved in the pathogenesis of OA (Dingle 1984, Hamerman 1984, Herman, Appel and Hess 1984, O'Byrne et al. 1984). The overall purpose of this work was to test the hypothesis that catabolin is produced by OA synovium and may be involved in the pathogenesis of OA. The specific aims are 1) to demonstrate that canine synovium is capable of producing catabolin 2) to show that it can stimulate canine articular cartilage to release GAG and 3) to determine whether or not experimentally osteoarthritic synovium in culture produces more catabolin than normal control synovium. Further, the progressive arthropathy in the experimental animals will be demonstrated by evaluation and grading of the gross and radiographic changes.
Chapter 2
MATERIALS AND METHODS
2.1 Materials

1-9 dimethylmethylene blue chloride was obtained from Serva Fienbiochemica, Heidelberg, Germany. Whale chondroitin sulphate (grade 3), retinol (vitamin A, synthetic, type X) and papain (type 4) were obtained from Sigma, St. Louis, MO., U.S.A.. Eagle’s minimal essential medium (MEM) and heat inactivated fetal bovine serum were obtained from Gibco Laboratory, ON. All other chemicals were reagent grade and obtained from Fisher Scientific Co., Vancouver, BC. Halothane and atropine were obtained from Clark Rogers, Abbotsford, BC. Ethanol was obtained from Schering, Pt. Claire, PQ. Nembutal was obtained from MTC Pharmaceuticals, Missisauaga, ON. Oxygen and nitrous oxide was obtained from Medigas, Vancouver, BC. Chloramphenicol was obtained from Roger STD, London ON. The porcine catabolin was kindly supplied by Dr. J.T. Dingle, Cambridge, England. Microtitre plates were purchased from Becton Dickinson, Los Angeles CA. Fresh bovine nasal cartilage was obtained from Intercontinental Packers, Vancouver. Foxhounds were purchased from the University of Oregon, Department of Animal Care, Portland, OR. Suture material was obtained from Davis and Geck, Vancouver BC.

2.2 Methods

2.2.1 Induction of Experimental Osteoarthritis

Osteoarthritis was induced in skeletally mature male American foxhounds by transection of the anterior cruciate ligament (ACL). Since dogs are quadrupeds, this ligament is properly called the cranial cruciate ligament, however in this paper the human term will be used. Preoperative anteroposterior and lateral radiographs were taken of both knees to ensure the joints were normal and the growth plates had
Materials and Methods

closed. The knees were examined for ligamentous stability using the anterior drawer test at 30° and 90° of knee flexion. This is a test for anterior cruciate ligament function. In an operating theater, after a prophylactic intramuscular injection of chloramphenicol 200 mg, general anesthesia was induced with Nembutal 30 mg/kg (sodium pentobarbital 65 mg/ml) and atropine (0.04 mg/kg), endotracheal intubation and ventilation with nitrous oxide and oxygen (2/1) and Halothane (1%). After a lateral arthrotomy between extensor digitorum longus and the patellar tendon of the right knee, the ACL was divided sharply in the midsubstance. A positive anterior drawer test was obtained at this time to ensure the ligament was transected. In the sham operated animals the arthrotomy was performed and a probe was passed behind the ligament. The joint was irrigated with normal saline and the capsule and synovium were closed with 2-0 Dexon, the superficial fascia with 3-0 Dexon and the skin was closed with 3-0 polypropylene sutures. A sterile dressing was applied. Skin sutures were removed ten days postoperatively by which time the animals had been partially weight bearing for one week. The animals were given the opportunity to exercise regularly.

2.2.2 Harvesting of Synovium

The animals were killed between two days and sixty four weeks with a lethal intravenous injection of 10 ml Euthansol (sodium pentobarbital 340 mg/ml). Both knees were examined for ligamentous instability. The knee joints were resected 'en bloc' without entering the joint. Anteroposterior and lateral radiographs were obtained of the specimens. Within one hour of sacrifice a lateral arthrotomy was performed and synovium was harvested from a defined anatomical area of
each joint including the medial and lateral gutters and the suprapatellar pouch. Care was taken to remove only the synovium and not the joint capsule. The genu articularis muscle inserts into the suprapatellar pouch and as much muscle tissue as possible was removed from the specimen. The specimen was kept moist by placing it into sterile saline at 25°C and tissue weight was obtained. The remainder of the joint was dissected and photographed. Specimens of meniscus, articular cartilage and synovium were taken for histological and biochemical analysis.

2.2.3 Synovial Culture

The synovium was washed three times in sterile saline and then minced into 0.5cm³ pieces and cultured in Eagle's minimal essential medium (MEM). The medium was prepared without serum, with penicillin (5000 I.U./ml), streptomycin (5000 ug/ml), amphotericin B (250 ug/ml) and buffered with 2.2ml of 7.5% sodium bicarbonate per 100ml of medium in a 5% CO₂ atmosphere and 37°C. Approximately ten ml of medium was used per gram wet weight of synovium. The medium was changed every two days for a total culture period of eight days. The supernatent medium was stored in sterile containers at -20°C. The synovium was discarded at the end of the culture period.

2.2.4 Purification of Catabolin

Catabolin was purified essentially as described by Dingle (1979). The samples of medium were thawed to 15°C and filtered with a 0.45 um filter. Ammonium sulphate was added to the sample to a concentration of 60% and mixed on rollers at 4°C for two hours. This was centrifuged at 17,000 RPM for 45 minutes. The pellet was discarded and...
the supernatant was made up to a 95% ammonium sulphate concentration and mixed on rollers for two hours at 4°C followed by centrifugation at 17,000 rpm for 45 minutes. The supernatant was discarded and the pellet was redissolved in 0.5ml of MEM with the addition of 5% heat inactivated fetal bovine serum (IFBS). The sample was dialyzed against 40 volumes of MEM with three changes of dialysate at 4°C. The volume of sample was then made up to 1.7ml with the addition of MEM with 5% IFBS. This fraction containing the catabolin sample was stored at -20°C.

2.2.5 Bioassay for Catabolin

Bovine nasal cartilage was obtained fresh from a local abattoir within four hours of slaughter. Small discs (2mm x 0.5mm) of cartilage were punched from the septum and washed in three changes of normal saline. The discs were pre-cultured in flasks in MEM with 5% IFBS at 37°C in a 5% CO₂ atmosphere for four days.

The discs were then placed in microtitre plates with the catabolin sample to be assayed and sufficient medium was added to make up a volume of 200ul with MEM and 5% IFBS. All assays were performed in quadruplicate with medium blanks and freeze thawed (three cycles) killed cartilage as negative controls. Retinol(3ug/ml) and porcine catabolin served as positive controls. The plates were cultured at 37°C and in a 5% CO₂ atmosphere.

After four days of culture the disc and medium were separated and the cartilage disc digested with papain containing 8.5 units of papain per ml of buffer (50 mM sodium acetate, ph 6.0, 10 mM cysteine hydrochloride and 50 mM EDTA at 60°C for 24 hours).
The concentration of sulphated glycosaminoglycans in the media and in the papain digest was measured using an automated version of the 1,9-dimethylmethylene blue chloride (1,9-DMB) dye binding assay. The originally described method (Humbel and Etringer 1974) was found to be unworkable because of precipitation of the dye complex and so the method of Farndale et al. (1982) was adapted to a Technicon Autoanalyser 1 system consisting of a sampler II with a digital electronic timer, proportioning pump II, a colourimeter and a chart recorder. The size of tubing was chosen to minimize the amount of sample. The transit time from the mixing coil to the colourimeter was less than two minutes. The flow diagram is shown in figure 1. A stable solution of 1,9DMB was prepared by stirring 12mg of 1,9-DMB with 5ml of 100% ethanol. After the addition of 2.0gm sodium formate and 2.0ml formic acid, the volume was made up to one liter with distilled water and stored at room temperature. The solution was stable for four weeks.

The calibration curves of different GAGs are shown in Figure 2. The colour yield of each was linear with concentration, but the intensity of metachromasia decreased in the order; chondroitin sulphate > heparin > dermatan sulphate > keratan sulphate. Hyaluronic acid to concentrations of 500 ug/ml did not give any colour yield. Albumin, at a concentration above 0.1 mg/ml was found to interfere with the assay, but this interference could be abolished by papain digestion. The concentration of GAG in the medium required a one to four dilution and in the papain digest of the cartilage disc a one to six dilution to fall within the standard curve of the whale chondroitin sulphate used (40 -800ug/ml). There was no significant interference under these conditions. The results are calculated as the ratio of the concentration of GAG in the medium to the sum of the
Materials and Methods

Figure 1. Flow diagram for 1-9 DMB assay

![Flow diagram for 1-9 DMB assay]

Figure 2. Absorbance vs concentration of different GAG using 1-9 DMB assay

![Absorbance vs concentration of different GAG using 1-9 DMB assay]
Materials and Methods

concentration of GAG in the medium and in the papain digest of the disc and expressed as % GAG released.

2.2.6 Technique and Analysis of Radiographs

The intubated animals were positioned with the X-ray tube one meter from the film. True anteroposterior and lateral projections were obtained of both knees by taping the hind limbs into position. The film used was Kodak X-OMAT TL. The technique used on the AP film was 200 mA and 90 KV for 0.4 seconds and the lateral film 200 mA and 80 KV for 0.25 seconds. The films were processed in a Kodak RPH-OMAT processor.

Osteoarthritis in general can be graded radiologically based on the system in the Atlas of Standard Radiographs of Arthritis (1963):

Grade 0: none

Grade 1: doubtful - doubtful narrowing of joint space and possible osteophytic lipping

Grade 2: minimal - definite osteophytes and possible narrowing of joint space

Grade 3: moderate - multiple osteophytes definite narrowing of joint space, some sclerosis and possible deformity of bone ends

Grade 4: severe - large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone end

However, specific changes occur in OA of the ACL deficient knee. Faegen et al.(1982) described the radiographic findings of the unsuccessfully repaired anterior cruciate ligament in man. The changes appeared as early as six months and showed sequential deterioration.
These radiographic signs appear in the order:

1/ Peaking of the intercondylar tubercles (lateral > medial)
2/ Spurring and hypertrophy of the intercondylar eminence
3/ Osteophytosis of the inferior pole of the patella
4/ Stenosis of the intercondylar notch
5/ Narrowing of joint space and buttressing osteophytes

Since these findings were also commonly seen in the dogs with surgically transected ACL they were incorporated into the radiological grading of osteoarthritis used in this work.

Grade 0: none

Grade 1: doubtful - doubtful narrowing of joint space, possible osteophytic lipping and peaking of the intercondylar tubercles (<2mm osteophytes)

Grade 2: minimal - osteophytes, possible narrowing of joint space and osteophytosis of the inferior pole of the patella (3-4mm osteophytes)

Grade 3: moderate - multiple osteophytes definite narrowing of joint space, some sclerosis, possible deformity of bone ends and stenosis of the intercondylar notch (5-6mm osteophytes)

Grade 4: severe - large osteophytes, marked narrowing of joint space, severe sclerosis, definite deformity of bone end (>6mm osteophytes)

2.2.7 Preparation and Grading of Gross Specimens

Once the synovium had been harvested the joint was dissected, gross pathological changes were noted and standard photographs were
taken. The distal femur was cleared of tendons, capsule and ligaments. Photographs were taken from the medial, lateral and weight bearing surface. The proximal tibia was similarly cleaned and photographed with the menisci in place and then after their removal.

Pathological changes of the articular surface can be graded using the technique described by Meachin (1972). He described three grades of changes in human articular cartilage using Indian ink staining. 

**Grade 1:** intact surface—these areas were normal in appearance and did not retain any ink;

**Grade 2:** minimal fibrillation—these areas appear normal before staining but retain the ink as patches of light grey;

**Grade 3:** moderate fibrillation—these areas were velvety in appearance and retained the ink as dark patches;

Since the specimens in this study did not have Indian ink applied because of concern about the effect of Indian ink on the biochemical studies and the lack of sensitivity to noncartilaginous changes, gross evidence of fibrillation was combined with the other features found in the experimental animals which included cartilage erosion, meniscal tears and osteophytes to grade the gross pathological degree of osteoarthritis for the purpose of this study.

**Grade 0:** none

**Grade 1:** doubtful—minimal discolouration, minimal fibrillation and no osteophytes

**Grade 2:** minimal—definite discolouration, small osteophytes and small erosions

**Grade 3:** moderate—large osteophytes, erosions and meniscal


2.2.8 Preparation of Histologic Material

Samples of articular cartilage and synovium were obtained at the time of joint dissection and stored in alcoholic formalin, made with 10 parts of 40% formaldehyde and 90 parts of 70% ethyl alcohol. The tissue was dehydrated, cleared, embbeded in parafin and then cut into 5u sections. The synovium was stained with Harris's hematoxylin for 5 to 10 minutes and then counter stained with nanoimino eosin for 1 minute. The tissue was then cleared and mounted. The articular cartilage was similarly fixed and was then stained with Weirgert's iron hematoxylin for 30 to 40 minutes, followed by 1/5000 aqueous fast green for 3 minutes and then counter stained with 1.0% aqueous safranin-O for 4 to 6 minutes. Similarly the section was cleared and mounted.
Chapter 3

RESULTS
3.1 Effect of Catabolin on BNS and Canine Articular Cartilage

The first stage of this work was to determine if normal canine synovium produced catabolin using bovine nasal cartilage for the assay. With a 95% ammonium sulphate precipitate of the canine synovial media stimulating bovine nasal explants living explants released 87% of the total GAG into the supernatant medium. Further separation using the 60-95% ammonium sulphate ppt. fraction eliminated most of the proteolytic enzymes known to be present in the 30-60% fraction (Cawston and Tyler 1979) and accentuated the difference between the live and dead explants (figure 3). The percent GAG release was linearly related to the amount of catabolin added to the culture up to approximately 80%. These findings are similar to Dingle et al. (1979).

In order for catabolin to be implicated in the pathogenesis of OA it must also be able to stimulate canine articular cartilage to release GAG. In the second part of this study with maximal stimulation of canine articular cartilage by canine catabolin there was 50% GAG release with some apparent proteolytic effect as evidenced by a 23% GAG release by the dead cartilage. Retinol, known to stimulate living chondrocytes to cause release of GAG from the matrix was used as a positive control (figure 4). Thus, canine synovium does produce catabolin which can stimulate GAG release from both bovine nasal septum and canine articular cartilage.

3.2 Radiographic Analysis

The first radiographic changes became apparent 12 weeks postoperatively with progressive changes up to 64 weeks. The earliest
**Results**

**Figure 3.**

% GAG released vs ammonium sulphate precipitation using bovine nasal septum

![Graph showing % GAG released vs ammonium sulphate precipitation.](image)

**Figure 4.**

Canine Articular Cartilage

![Graph showing % GAG released for different conditions.](image)
Results

Changes were small osteophytes on the inferior pole of the patella, followed by osteophytes on the posteromedial tibial plateau. At 32 and 64 weeks all animals had large osteophytes on both the femoral and tibial condyles. As in humans with anterior cruciate ligament deficiency (Feagin et al. 1982), stenosis of the intercondylar notch was also a feature in the dog. The post-mortem films were non-weight bearing therefore joint space narrowing could not be assessed. The radiographs were graded according to the classification outlined in materials and methods and presented in figure 5. More animals were available for radiographic analysis than are in the catabolin bioassay. Our results were entirely in agreement with Marshall and Olsson (1971), who noted progressive enlargement of osteophytes in the same areas up one year post ligament section. Figure 6 shows the lateral radiographic appearance of a normal knee and figure 7 an arthritic joint 32 weeks after ligament section with arrows highlighting the osteophytes. Radiographically the animals developed changes consistent with OA starting at 12 weeks and progressing to 32 weeks.

3.3 Pathological Assessments

Changes were apparent grossly within the first few weeks with discolouration of the cartilage. By four weeks there were visible erosions on the lateral tibial plateau and chondrophytes particularly along the femoral trochlea. At four to eight weeks well formed osteophytes were visible on the femoral condyles, tibial plateaus and the inferior pole of the patella. Erosions were seen on the tibial plateaus and on the medial femoral condyles. Meniscal tears usually on the medial side were occasionally seen at 16 weeks. By 64 weeks the changes already
Results

Figure 5.

Grading of radiographs of OA animals

X-ray grade of OA

Weeks
Figure 6. - Lateral roentgenogram of a normal knee
Results

Figure 7.- Lateral roentgenogram of an OA knee 32 weeks postoperatively.
The arrows indicate osteophytes.
described showed progression. The osteophytes on the postero-medial tibia and the trochlear groove were particularly impressive and the menisci were frequently torn with displaced bucket handle tears. Each joint was graded using the system outlined in the materials and methods and presented in figure 8. Marshall and Olsson (1971) killed their animals between six and eight months and a second group between 20 and 23 months. They noted the development of osteophytes and a large number of meniscal tears, particularly of the bucket handle variety. Figure 9 shows a normal distal femur and figure 10 an arthritic distal femur 32 weeks postoperatively. In summary the gross specimens showed changes in the articular cartilage which were grossly visible between one and four weeks and deteriorate progressively up to 64 weeks. These changes were consistent with OA.

3.4 Histological assessments

The histological grade has not been formally assessed yet, but some general comments can be made. At 32 weeks post ligament section, the articular cartilage on the femoral condyles when stained with safranin O showed loss of superficial staining. Safranin O is a cationic dye which binds to anionic proteoglycans. Loss of superficial staining would indicate loss of proteoglycans. The tibial plateaus showed more extensive changes with fissuring of the cartilage and clumping of the chondrocytes and a decrease in staining around some of the chondrocyte lacunae. The synovial linings from arthritic joints were hypertrophic with prominent villi, but lacking inflammatory cells. Figure 11 shows normal synovium and figure 12 the hypertrophic OA synovium.
Figure 8.- Pathological grading of gross specimens.
Figure 9. Normal distal femur

Figure 10. Osteoarthritic distal femur 32 weeks postoperatively.
Results

**Figure 11.** - Photomicrograph with H/E stain of normal synovium.

**Figure 12.** - Osteoarthritic synovium, H/E stain 32 weeks postoperatively.
3.5 Catabolin Bio-assay

In each set of experiments living and dead bovine nasal cartilage stimulated with either porcine catabolin or retinol and unstimulated blanks were used as controls. Control catabolin samples were obtained from cultures of synovium from the left unoperated of each animal.

The synovial media was changed every two days for a total culture period of eight days, yielding four fractions from each knee. Figure 13 represents a fairly typical example of a four week animal. The solid bars represent the right operated OA knee and the hatched bars the left control knee. The catabolin sample from the right knee clearly caused more GAG release than that from the left knee. For analysis the first fraction was discarded as was done by Dingle and collaborators (1979) since the results were variable and likely represented the trauma of excision and the initiation of culture. The remaining fractions were added after subtracting the baseline release from unstimulated controls and expressed as a ratio of GAG release from the right to left knees.

The results from the seven animals operated on at one, four and 32 weeks are illustrated in figure 14. The ratio between the right and left knees in each case being greater than one indicating the synovial media from the right OA knee producing more catabolin than the left control knee. When analysed using the Wilcoxon rank sum test this was significant to p < .05. When catabolin production by the OA synovium was normalized for the amount of synovial hypertrophy by dividing by the mass of synovium there was no statistically significant difference between the OA synovium and control synovium (figure 15).

In three sham operated animals at one, four and 32 weeks depicted
in figure 16, two animals showed no difference and in a third animal the synovium from the right knee showed an increase in catabolin production. When normalized per gram of synovium in culture this relationship did not change.

The addition of cortisol succinate in a concentration of 1 ug/ml of medium was tested on live bovine nasal cartilage with and without the addition of canine catabolin. The addition of cortisol seemed to inhibit the ability of catabolin to cause GAG release with an average decrease of 20% GAG release in quadruplicate samples (figure 17).
Figure 13.

Dog 4-2 % GAG Release

Sample fraction in days

% GAG Released

Figure 14.

Ratio of right to left knees of GAG released in experimental OA animals

Ratio

Experimental animals
Figure 15.

Ratio of right to left knees of GAG released per gram of synovium in experimental OA animals

Figure 16.

Ratio of right to left knees of GAG released in sham operated animals
Figure 17.

Effect of cortisol succinate (1 ug/ml) on catabolin bioassay using BNS

- % GAG released
- live
- live
- live
cortisol
plus
plus
- catabolin
cortisol
- catabolin
Chapter 4
DISCUSSION
Discussion

In the experimental model of OA used there were changes of progressive arthropathy similar to those of spontaneous OA. Grossly these changes were evident between one and four weeks postoperatively and progressed up to 32 weeks. Radiographic changes became apparent 12 weeks postoperatively with progression up to 64 weeks postoperatively. While there were destructive changes of articular cartilage and menisci there was also evidence of hypertrophy of cartilage, synovium and capsule. Marshall and Olsson (1971) also found proliferative and degenerative changes. They felt that by one year the capsule thickened, the joint stabilized and the osteophytes stopped enlarging. Our results showed little progression between 32 and 64 weeks supporting this hypothesis. However, they noted only occasional degenerative changes of cartilage, whereas we consistently found degenerative changes which increased with time. While the total proteoglycan content of articular cartilage declines in early OA (McDevitt et al. 1977, Muir 1977), Mankin and Lippiello (1970) and McDevitt and Muir (1976) have shown an increase in chondrocyte synthetic activity in human and experimental OA respectively. These results suggest that in OA catabolic and anabolic processes both occur. In order for catabolic synovial factors to be involved in the pathogenesis of OA these findings would have to be explained.

The purpose of this work was to test the hypothesis that catabolin is produced by OA synovium and may be involved in the pathogenesis of OA. To do this we proposed to 1) demonstrate that canine synovium is capable of producing catabolin 2) to show that it can stimulate canine articular cartilage to release GAG and 3) to demonstrate whether or not
experimentally osteoarthritic synovium in culture produces more catabolin than normal control synovium. Our in vitro results showed that canine synovium produced catabolin which could stimulate living bovine nasal cartilage and canine articular cartilage to release GAG. These findings are in agreement with others (Dingle et al. 1979, Dingle 1984, Saklatvala 1981). The finding that catabolin stimulated articular cartilage did not release as much GAG as bovine nasal septum suggests that the GAG in articular cartilage is more tightly bound and reflects the different functions of these tissues. The GAG release from articular cartilage was not as predictable as bovine nasal cartilage. The inconsistency may be related to the variable ages of the animal or to the different joints harvested.

For the third part of this work a defined anatomical area of synovium was harvested from experimentally induced osteoarthritis, using the canine anterior cruciate ligament section as the model. The synovium was grown in tissue culture and assayed for catabolin. The results were compared against unoperated and sham operated control synovium. The total amount of catabolin measured from cultures of experimentally induced OA synovium was statistically significantly greater than that measured from unoperated control synovium. However, when this was calculated per gram of synovium there was no statistically significant difference. Unoperated control synovium in culture on a per gram basis produced as much catabolin as osteoarthritic synovium. The synovium from the four week sham operated animal produced more catabolin from the right than the left knee. There are not enough sham operated animals to analyse this finding for significance and further work is needed.
The experimentally osteoarthritic synovium was obtained one, four and thirty two weeks post ligament section. It is possible that catabolin is active only at certain times in OA. McDevitt and Muir (1976) noted changes in synovial hypertrophy one week post ligament section and our thirty two week synovial histology showed hypertrophy. If catabolin was active in OA it would likely be during the period of synovial hypertrophy.

Catabolin, as the name implies, stimulates catabolism. Its mechanism of action is unknown, but it is thought to stimulate living chondrocytes to secrete proteolytic enzymes into the pericellular environment. Dingle (1984) has recently shown that catabolin not only stimulates cartilage matrix degradation, but also inhibits chondrocyte synthetic activity in vitro. As yet there is no firm evidence supporting an in vivo role for catabolin in the pathogenesis of OA despite an increase in catabolin production by OA synovium. Dingle has suggested that the inhibition of synthesis combined with local degredation may make the articular cartilage more susceptible to mechanical damage, or that osteoarthritic articular cartilage is more susceptible to the actions of catabolin. However, the dramatic 80% GAG release in vitro is unlikely to occur in vivo. Our findings, that OA synovium and unoperated control synovium produced the same amount of catabolin per gram of synovium suggests that if catabolin is active in the pathogenesis of OA it related to the degree of synovial hypertrophy. The magnitude of the catabolin stimulated GAG release in our in vitro work suggests that the action of catabolin is predominantly degradative rather than inhibiting synthesis.

The effect of corticosteroids on catabolin has been studied by Saklatvala (1981) and Steinberg et al. (1983). Our preliminary findings
would agree with Steinberg et al. (1983) that the addition of cortisol inhibited the action of catabolin on cartilage. The deleterious effect of intra-articular corticosteroids are well known (Salter et al. 1967, Bentley and Goodfellow 1969).

It is possible that an actual difference between the amount of catabolin produced by OA and control synovium was missed. There are several sources for such an error in the technique used. The ammonium sulphate precipitation method could not be checked for recovery, however all samples were treated identically. There was considerable variability in the bioassay results between different bovine nasal septi. Therefore comparisons were only made between the right and left knee which were cultured under the same conditions using a single bovine nasal septum. The conditions of culture were somewhat arbitrary and may not have been ideal. While the volume of medium per gram of synovium (10ml/gm) was less than that used by others (25 ml/gm) (Saklatvala and Dingle 1980) the medium was changed every two days as opposed to every four days.

In vitro results do not necessarily reflect what is happening in an arthritic joint and therefore further in vivo study is needed to delineate the role of catabolin in OA. Purification of catabolin to homogeneity is needed in order to distinguish it from other intercellular factors. This would aid in determining the site of action of catabolin and the factors controlling catabolin synthesis.


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