THE ROLE OF KIAA1199 IN CROHN’S DISEASE

by

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*We also certify that written approval has been obtained for any proprietary material contained therein
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>18S</td>
<td>18S subunit of ribosomal RNA</td>
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<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>Fb</td>
<td>Fibroblasts</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>HA</td>
<td>Hyaluronan</td>
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<td>HADA</td>
<td>Hyaluronan Degradation Assay</td>
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<tr>
<td>HAS</td>
<td>Hyaluronan Synthase</td>
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<td>HYAL</td>
<td>Hyaluronidase</td>
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<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ND</td>
<td>Non-Diseased</td>
</tr>
<tr>
<td>RHAMM</td>
<td>Receptor for HA Mediated Motility</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
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The Role of KIAA1199 in Crohn’s Disease

Abstract

by

ARTIN SOROOSH

Crohn’s Disease (CD) is a chronic inflammatory disease of the gastrointestinal tract in which fibrosis, the excess deposition of extracellular matrix (ECM), frequently develops in affected areas and causes organ impairment. One ECM component, hyaluronan (HA), is a glycosaminoglycan which, when fragmented, becomes a well-documented pro-inflammatory molecule. A recent study has identified the novel protein KIAA1199 to be involved in HA degradation. We hypothesized that expression of KIAA1199 is altered in CD colon fibroblasts when compared to non-diseased (ND) colon fibroblasts and affects HA degradation in CD cells. Our data indicates elevated levels of cellular and extracellular KIAA1199 protein in CD colon fibroblasts compared to ND. In addition, silencing of KIAA1199 results in decreased HA degradation. Furthermore, our data indicates that the pro-inflammatory cytokine interleukin-6 upregulates KIAA1199 in the ECM. These data together suggest that in CD, KIAA1199 is elevated and plays a role in degrading HA.
CHAPTER 1: INTRODUCTION

1.1 CROHN’S DISEASE

Crohn’s Disease (CD) is a chronic inflammatory disease of the gastrointestinal tract. CD is part of a group of diseases known as Inflammatory Bowel Disease (IBD). This umbrella term includes another well-known disease, Ulcerative Colitis (UC).

Common symptoms of IBD include diarrhea, vomiting, abdominal pain, bleeding, and weight loss (1).

Crohn et al. first characterized CD in 1932 as an inflammatory and necrotizing disease of the ileum (2). The incidence of the disease has increased since then, with higher rate of the disease in developed countries of Europe and the United States (3). CD commonly presents in patients at a young age and leads to a lifetime of complications that can result in necessary surgical intervention (4).

CD is unique in that it can affect any part of the GI tract, but most commonly affects the terminal ileum, colon, and/or the perianal region (5). Additionally, inflammation specifically in CD is transmural and manifests in discontinuous patches, called skip lesions, encompassing the affected area (6).
1.1.1 Crohn’s Disease Pathology

Though the disease is of an unknown etiology, it is thought that a number of elements are involved in the development of CD in humans. These factors include genetic predisposition and environmental influences.

The role of genetic factors in CD is evident from statistics showing its familial aggregation (7). There is a high rate of CD among monozygotic twins if one twin has CD in addition to a high rate of family history in CD patients (8). Genetic metanalyses were done to identify gene variants that lead to susceptibility to CD, with over 30 genes originally identified, and now up to 100. In general, these mutations are thought to cause functional deficiencies in the innate immune system. In the gene with the strongest association, variants of the intracellular pattern recognition receptor NOD2/CARD15 were linked to increased CD susceptibility (9, 10). NOD2/CARD15 acts an intracellular receptor for peptidoglycans from bacteria, specifically muramyl dipeptide molecules. They react by stimulating an immune response in the infected epithelial cell (11). Many other genes linked to CD susceptibility are involved in innate immunity and protection (8).

A prevalent environmental factor involved in CD is smoking. Some studies investigating the link between smoking and CD have indicated that a higher proportion of CD patients were smokers, and those patients that smoked were at a higher risk of developing worse outcomes than patients that did not smoke (12, 13).
The link of increased CD prevalence to developed countries may be explained by studies linking dietary differences between CD patients and non-diseased (ND) patients. High intake of refined carbohydrates was associated with manifestation of CD. The consumption of sugar could be responsible for changes in gut microbiota that negatively affect the intestinal lining and leads to CD (14). Other dietary differences seen in CD patients include increased intake of dietary fat, specifically levels of omega-6 fatty acids, and increased intake of animal protein (15).

1.1.2 Major Complications of Crohn’s Disease

Crohn’s disease can lead to many serious intestinal and extraintestinal complications in patients over time (16).

The extraintestinal manifestations of Crohn’s disease can appear in different tissues of the human body. CD patients are susceptible to reactive skin eruptions including erythema nodosum and pyoderma gangrenosum in addition to skin disorders that are directly related to the inflammatory process of the disease (17). Ocular involvement is also common in CD patients, with uveitis and conjunctivitis often manifesting with degenerative ocular clinical symptoms including pain, photophobia, loss of visual acuity, and possible blindness (18, 19). In addition, patients with CD have a higher risk of developing thrombosis, bone complications, arthritis, and amyloidosis (16, 20, 21).
The intestinal complications of CD stem from the chronic and transmural nature of its inflammation in the intestines. The damage caused to the gastrointestinal lining can cause a variety of obstruction and absorption issues including strictures, chronic malabsorption leading to chronic malnutrition, and anal fistulae (22, 23, 24). All of these complications lead to organ impairment and may require surgical intervention to alleviate.

The penetrating nature of inflammation in CD is important to note because of arguably the most significant intestinal complication of CD, fibrosis. Fibrosis, characterized as the accumulation of scar tissue in the gastrointestinal lining that leads to thickening and hardening of the gastrointestinal tract, is thought to be responsible for the aforementioned intestinal complications of CD (25). While inflammation can be controlled using immune suppressing medication, there is no effective treatment for fibrosis outside of surgical intervention. In many cases, once fibrosis has begun, anti-inflammatory treatments will not affect it and eventually, the patient will need surgical resection (26).

1.1.3 Fibrosis

Fibrosis can be viewed as a form of uncontrolled wound healing. It is characterized by excess deposition of extracellular matrix proteins with the intent to heal injured tissue. This process, when uncontrolled, eventually leads to scarring of the tissue and organ malfunction. Fibrosis can occur in a variety of different tissues and organs in the human body, including the lungs, kidneys, liver, cardiovascular system, and
the intestines (26). Chronic fibrosis in general has been attributed to 45% of deaths in the developed world (27).

Fibrosis is thought to be a response to chronic inflammation. Currently accepted models suggest that the recruitment of inflammatory cells to the tissues cause the release of reactive oxygen species and pro-fibrogenic cytokines (26). The penetrating nature of the inflammation in CD likely causes fibrosis in the entire thickness of the area that is affected (25). Often, this transmural fibrosis leads to strictures and obstruction in the intestines that require surgical intervention to relieve (28).

The progression of fibrosis stems from cells of mesenchymal origins that become activated and deposit ECM. These mesenchymal cell types include stellate cells (29), bone marrow-derived cells (30), fibrocytes (31), pericytes (32), fibroblasts, and myofibroblasts (25). In addition, non-mesenchymal epithelial and endothelial cells can differentiate into mesenchymal cells in a process called epithelial/endothelial to mesenchymal transition, creating fibrogenic cells (33, 34).

In intestinal fibrosis, however, fibroblasts are the main effector cell involved. During intestinal fibrosis, many of the aforementioned mesenchymal cells differentiate into the activated version of fibroblasts, called myofibroblasts, responsible for the increase in ECM deposition in the gastrointestinal lining (25).
The regulation of myofibroblast activity is important in the pathogenesis of fibrosis. Fibroblasts can be recruited and activated by a number of different biological mediators including a variety of growth factors, cytokines, and chemokines. Many of these regulatory factors are produced by the cells involved in inflammation (25,27).

A major growth factor involved in tissue fibrosis is transforming growth factor beta (TGF-β), specifically the TGF-β1 isoform (27). TGF-β1 is produced by and can act on a variety of cells, and is increased in the colonic mucosa of patients with IBD (35, 36). TGFβ1 can directly stimulate fibroblast proliferation and upregulate other fibrogenic cytokines (26, 37).

Part of the inflammatory response in humans involves T helper cells. T helper cell type 1 (Th1) is involved in the preliminary inflammatory response that is involved in onset and/or perpetuation of CD (5). However, the Th1 inflammatory response secretes numerous anti-fibrotic cytokines including interferon-gamma (IFNγ) (25, 38, 39). The T helper cell type 2 (Th2) inflammatory response includes the secretion of many pro-fibrotic cytokines including interleukin 4 (IL-4) and interleukin 13 (IL-13) (27). Among other profibrotic functions, IL-4 was found to stimulate collagen synthesis in fibroblasts (40) and IL-13 was linked to the induction and activation of TGF-β (41). Some mouse studies suggest that late stage IBD involves the Th2 mediated cytokines (42). These data together suggest that the Th2 mediated inflammatory response drives fibrosis in the later stages of CD, but the distinct roles of Th1 and Th2 in CD are still unclear (25).
The fibrotic thickening and hardening of the intestines is caused by the excess deposition of ECM. Normally, the ECM is composed of interlinked and interactive proteins, proteoglycans, and glycosaminoglycans that surround cells (43). The components of ECM interact with the cell and exterior in numerous capacities. The ECM is involved in cell structure, signaling, migration, and other functions that are integral for the survival of cells (43). In fibrosis, the ECM is remodeled into a more rigid structure, especially by elevated levels of collagen as well as numerous other proteins and glycosaminoglycans, including hyaluronan (HA) (44).

1.2 HYALURONAN

Hyaluronan (HA) is a glycosaminoglycan (GAG) comprising of repeating disaccharides of n-acetylglucosamine and glucuronic acid connected by β-glycosidic bonds (45, 46). HA is non-sulfated, lacks a protein core, and can range in size from 4,000 to 10^7 Da (47). It is a common component of extracellular matrices and is found throughout the human body (48).

HA was first discovered as a polysaccharide isolated from the vitreous humor of cow eyes in 1934 (49). HA, with its large size range, has many diverse functions which include cell lubrication and hydration, shock absorption, and wound healing in its larger molecular weight forms, and inflammation, regulation of cell-cell and cell-matrix adhesion, and promotion of proliferation in its fragmented lower molecular weight forms (47, 50, 51). Elevated levels of HA in serum are a disease marker, especially for liver inflammation. Due to either increased production from unhealthy
tissue or impaired clearance by the liver in diseased states, HA in serum is measured to indicate disease and at times, its progression as well (52). Some of these pathological conditions include liver disease, inflammatory diseases, and certain cancers (53).

1.2.1 Hyaluronan Metabolism

Hyaluronan is created and turned over at a rapid rate (47, 54). HA does not have a protein core and is not synthesized in the Golgi apparatus, like other GAGs. Hyaluronan is created at the cytosolic cellular membrane and secreted outside the cell by a family of enzymes called hyaluronan synthases (HAS) (55, 56). There have been three identified mammalian HAS genes: HAS1, HAS2, and HAS3. These enzymatically distinct proteins consist of integral membrane proteins spanning the parts of the cytosolic cell membrane (56, 57). As these enzymes create HA, it is simultaneously secreted out of the cell into the ECM through the multidrug resistance-associated protein 5 (MRP5), a member of the ATP-binding cassette (ABC) transport protein superfamily (58). After leaving the cell, HA is incorporated into the matrix where it binds with HA binding proteins, or hyaladherins, in order to go about its function (59).

There are a few theorized mechanisms of HA degradation, both enzymatic and non-enzymatic. Some non-enzymatic reactions that degrade HA include acidic and alkaline hydrolysis, ultrasonic degradation, and thermal degradation. However, these are very unlikely at physiological states (51). However, one enzyme-
independent method of HA degradation is by way of reactive oxygen species. Inflammatory cells can produce oxygen radicals (51, 60), which then have the ability to degrade HA (61). In regards to enzymatic methods of HA degradation, the most well studied theory involves the hyaluronidase (HYAL) family of enzymes.

The human genome encodes six hyaluronidase-like sequences, however two members of the family, HYAL1 and HYAL2, are the major players in HA degradation in human cells (47, 51, 62). HYAL2 is an acid active, glycosylphosphatidylinositol (GPI)-linked cell surface protein (63). Degradation via HYAL2 takes place at the cell surface and, in some circumstances, is dependent on the cell surface HA receptor CD44 (47, 64). In some cells, the HA-CD44 interaction activates sodium-hydrogen exchange to create an acidic microenvironment for the proper function of HYAL2 (65). HYAL2 degrades large HA bound to CD44 into small fragments, which are then internalized into the cell via early endosomes and further degraded by the acid-active HYAL1 enzyme in the lysosome (45, 47).

HA exists in a wide range of sizes. The functionality of the GAG is generally different at the two general size ranges, high molecular weight (1-10 MDa) and fragmented (<100 kDa) (45).

Large molecular weight HA in the ECM is generally beneficial for cell structure and stability. It is known to be anti-inflammatory and anti-angiogenic and promotes tissue integrity, hydration, and quiescence (45, 66, 67). An additional role of high
molecular weight HA is its presence in a cable form at sites of inflammation. Commonly called “cables,” they cross-link and promote interaction with non-activated mononuclear leukocytes (45, 68).

HA fragments, on the other hand, have been heavily implicated in inflammatory, proliferative, and angiogenic processes (45).

1.2.2 **Hyaluronan and Inflammation**

Hyaluronan has been implicated in IBD, with studies linking it to the initiation of chronic inflammation in mouse models (69). The specific mechanisms whereby HA induces inflammation are widely focused on HA fragments (45).

Degraded HA has been widely studied in inflammatory processes, and has been shown to be pro-inflammatory, pro-angiogenic, and immunostimulatory (51). HA fragments interact with different receptors in order to stimulate a variety of inflammatory, angiogenic, and proliferative responses.

The receptor CD44 is a well known HA receptor present on most cells. As mentioned previously, CD44 is involved with HYAL2 in degrading large molecular weight HA and internalizing the resulting fragments. As part of the immune response, CD44 is present on a variety of cell surfaces, including macrophages, neutrophils, eosinophils, and lymphocytes (70). Ligation of fragmented HA to CD44 on these cells produces a variety of pro-inflammatory reactions, including production of cytokines,
cell proliferation, and angiogenesis (71). For example, on macrophages, the CD44-HA fragment reaction triggers an intracellular pathway that leads to the activation of the transcription factor, nuclear factor-κβ (NF-κβ) (72). NF-κβ activity induces the production of a variety of inflammatory molecules, essentially activating the macrophage (71, 73). The CD44-HA inflammatory interaction has been implicated in several inflammatory diseases due to its widespread function in the immune response.

Certain toll-like receptors (TLR) are also well known HA receptors involved in inflammation. TLRs are a family of transmembrane proteins with downstream signaling pathways that are implicated in inflammatory responses. TLRs are known to bind to a range of microbial products and also recognize other inflammatory related ligands (74). Two specific members of the TLR family, TLR2 and TLR4, are noted HA receptors. Studies have suggested that HA fragment signaling via TLR2 and TLR4 induces pro-inflammatory reactions (75). Studies have found the TLR2- and TLR4-HA fragment interaction to be involved in numerous pro-inflammatory reactions including NF-κβ activation (76), macrophage chemotaxis (77), leukocyte maturation (78) and the production and release of pro-inflammatory cytokines from numerous cell types (78, 79, 80).

The receptor for HA mediated mobility, or RHAMM, is a HA receptor that promotes cell migration and proliferation (81, 82). RHAMM is not specifically associated with
any inflammatory events, but studies have implicated RHAMM to angiogenesis and migration of endothelial cells (83).

In all, there is overwhelming evidence implicating HA fragments as important pro-inflammatory signaling molecules in a variety of different facets. However, the mechanisms of HA fragmentation are not well understood throughout the human body, leaving room for further research into that process.

1.3 KIAA1199
KIAA1199 is a novel protein of unknown function. It consists of 1361 amino acids with a cleavable n-terminal signal sequence (84). It was first identified as an inner ear protein, whose mutations were linked to non-syndromic hearing loss (85). Further studies on KIAA1199 focused on its presence in numerous cancers. Studies have shown upregulation of KIAA1199 in gastric cancer (86), breast cancer (87), and colorectal cancer (88). The protein has been linked to increased tumor proliferation and invasiveness (86, 87). Additionally, the KIAA1199 gene was identified as a possible target for the Wnt signaling pathway and may be a regulatory part of the pathway (88, 89). Localization of the protein in colon cancer cell lines uncovered its presence in the endoplasmic reticulum and cell membrane (90).
1.3.1 **KIAA1199 and Hyaluronan**

In humans, KIAA1199 is expressed in many tissues, at the highest level in the brain (91). Coincidentally, the brain does not express the HYAL2 enzymes even with the tissue's high levels of HA. The first studies linking KIAA1199 to HA degradation were done by Yoshida *et al* (92). They were able to show the protein’s specificity to glycosaminoglycans. Additionally, the group was able to show the dependence of HA degradation on KIAA1199 by silencing the protein in dermal fibroblasts and showing the cell’s inability to degrade HA following siRNA treatment. Furthermore, the group also silenced HYAL2 and CD44 and revealed that the cells were still able to degrade HA (92).
CHAPTER 2: EXPERIMENTAL DESIGN, MATERIALS, AND METHODS

2.1 CELL CULTURE

The *in vitro* cellular model consisted of primary cells derived directly from surgically removed human colon specimen of CD or non-IBD patients, provided from the Department of Surgical Pathology at the Cleveland Clinic (Cleveland, OH, USA) as previously described by Rutgeerts et al (93). Fibroblasts were isolated using the explant method previously described by Strong *et al*. The tissue is stripped of its mucus by physical blotting and its mucosa is cut into strips. These strips are then treated with dithiothreitol (DTT), and then penicillin-streptomycin-amphotericin B antibiotic solution (PSF) in Hank's Balanced Salt Solution (HBSS). The strips are then minced into small pieces and placed on etched petri dishes to allow for outgrowth of fibroblasts from the colon mucosa (94).

Once grown enough to populate the majority of the dish, the cells are detached using 0.05% trypsin – 0.53 mM EDTA solution and then transferred to a large growth dish. All fibroblast detachment with the purpose of splitting is done with this trypsin-EDTA solution.

Human intestinal fibroblasts are grown in media consisting of the following:
Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal bovine serum (FBS), 2% L-glutamine, 1% PSF, and 2.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). All cells are incubated at 37°C in a humidified chamber containing 5% CO₂.
2.2 HARVESTING CELLULAR COMPARTMENTS

For the retrieval of whole cell lysates, extracellular matrix, and conditioned media, fibroblasts were harvested at two time points of growth, 3 days and 13 days. Giving the fibroblasts three days to grow allows them to become confluent in the dish that they are being grown. Adding 10 more days leads to increased deposition of ECM by the fibroblasts. For all plating of fibroblasts for experimental growth, cells are counted with a haemocytometer for equal starting cell numbers in the dishes that they are plated in. In a 10mm well of a 6-well plate, ~150,000 cells are plated. In a 50mm petri dish, ~800,000 cells are plated.

For retrieval of whole cell lysate, the fibroblasts were grown for the allotted growth time, then washed twice with cold phosphate buffered saline (PBS). The cells were lysed using the following lysis buffer: 0.5% NP-40, 1mM EDTA, 10% Glycerol, 300mM NaCl, 50mM Tris-HCl pH 7.6, and freshly added protease inhibitor for mammalian tissue P8340 (Sigma-Aldrich) (95). The lysis buffer was added to the plate and the cells were scraped off into the buffer using a cell scraper (Fisher Scientific). The lysate was then pelleted at 1600 g at 4°C for 15 minutes, and the insoluble portion was removed. The samples were prepared for immunoblot by adding Laemmli running buffer and DTT as a reducing agent. The samples were then heated at 95°C for 5 minutes.

The isolation and harvesting of ECM was based on a procedure previously described by Bonnefoy et al. After allowing the experimental growth time, the cells were
washed once with HBSS and incubated with 0.5% Triton-X 100 at 37°C for 10 minutes. After removal of the detergent, the dish was incubated with 0.025N ammonium hydroxide for 2 minutes at room temperature. Afterwards, the dish was washed 4 times with HBSS with calcium and magnesium. The matrix was then scraped up in lysis buffer containing 80mM Tris, 10% glycerol, and 3% SDS (96). To prepare the samples for immunoblot, 0.05% bromothenol blue and DTT was added, and the samples were heated at 95°C for 5 minutes.

2.3 IMMUNOBLOT

The total protein concentration of all samples designated for immunoblot were measured prior to the protein separation. Two protein assay methods were utilized: Bradford (Bio-Rad) and BCA (Pierce). Both were used following the manufacturer’s instructions.

Immunoblotting was done via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We used precast tris/glycine polyacrylamide gel at a 4-15% gradient (Bio-Rad). Samples were run through gels at 125V for 1 hour. The separated proteins in the gel were transferred onto a PVDF membrane using a semi-dry transfer apparatus from Bio-Rad. After transfer, the membrane was blocked with 5% milk in Tris-buffered saline with 0.1% tween-20 (TBST). All membrane-washing steps were done with TBST. Visualization of protein bands was accomplished using ECL prime chemiluminescent development (GE Healthcare).
To quantify the resulting bands of chemiluminescent signal, the films were scanned with the ImageScanner III and densitometry was done with the ImageQuantTL version 7.0 software package (GE Healthcare).

Antibodies used include: Rabbit anti-KIAA1199 (Proteintech), Rabbit anti-GAPDH (abcam), Rabbit anti-HYAL2 (abcam), and Donkey anti-Rabbit (abcam). Primary antibody incubation was done overnight at 4°C. Secondary antibody incubation was done for 1 hour at room temperature.

2.4 QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

To quantify mRNA levels, fibroblasts grown for the desired amount of time were lysed and collected for qPCR analysis using the Cells-to-cDNA kit (Invitrogen) following the manufacturer’s instructions.

2.5 IMMUNOHISTOCHEMISTRY

Fibroblasts for IHC were first plated on a glass coverslip in a 10mm well. After the experimental growth time (3 or 13 day), the cells were washed twice with HBSS. The slip was then removed from it’s well, and placed into a clean 10mm well and covered with 100% cold methanol. The 6-well plate was then placed in the freezer for 10min to permeabilize and fix the cells on the slip. The methanol was removed from the plate thereafter and the slip was allowed to dry.
To stain for KIAA1199, the slip was blocked in HBSS and 2% FBS for 1 hour, then incubated overnight at 4°C with anti-KIAA1199 antibody (1:100) in HBSS with 2% FBS. The next day, after washes, the slip was then incubated with AlexaFluor 568 Goat Anti-Rabbit antibody (abcam) at a 1:1000 concentration for 1 hour at room temperature. The slip was mounted onto a microscope slide with Vectashield with DAPI mounting medium (Vector Labs).

For staining of human colon tissue sections, paraffin-embedded blocks of colon tissue from CD and non-IBD patients were sectioned onto microscope slides and deparaffinized. The antibody incubation mimics the method described in the previous paragraph.

Both wide-field fluorescence microscopy and confocal microscopy were utilized to obtain images of KIAA1199 presence in colon tissue and colon fibroblasts. The wide-field fluorescence microscope was a Leica DM upright microscope, with the images taken using Image ProPlus acquisition software. The confocal microscope was a Leica TCS-SP upright microscope with Leica confocal software.

2.6 HYALURONAN DEGRADATION ASSAY (HADA)

HADAs were used to determine cellular HA degradation capability under certain conditions. Colon fibroblasts were treated with siRNA for KIAA1199 or HYAL2 by way of electroporation and then incubated with biosynthesized high molecular weight HA.
The siRNA used were chemically synthesized 25 base pair oligosaccharides previously employed by Yoshida et al. (92). Non-silencing control siRNA, called scramble siRNA, was used as a negative control. All siRNA was purchased from Invitrogen.

The conditions of the cells in this experiment were as follows: KIAA1199 siRNA without electroporation, HYAL2 siRNA without electroporation, Scramble siRNA, KIAA1199 siRNA, and HYAL2 siRNA. Each treatment had a duplicate to include a non-HA treated control. For each condition, ~300,000 fibroblasts were treated and plated on 10mm wells of 6-well plates with HIF media and incubated at 37°C and 5% CO₂.

Following one day of growth, the media was replaced with modified HIF media with 1% FBS. The wells designated for HA treatment were given 2ug/mL biosynthesized HA of 1000 kDa size (Hyalose). The cells were allowed to incubate for 2 more days.

After two days, the media was collected and the cells were harvested as mentioned above. The media was then treated with .25 mg/mL Proteinase K (Roche Applied Sciences) at 60°C overnight. The media was then heated to 95°C to inactivated the enzyme.

To measure the size of HA in the resulting media, agarose gel electrophoresis was used. The samples were prepared with sucrose-TAE-Bromophenol blue added. The
gel was made with 0.5% agarose in tris-acetate-EDTA (TAE). After overnight equilibration in TAE, the wells of the gel were loaded with sample. The sample was separated in the gel by electrophoresis at 30V for 30 minutes, then 60V for 3.5 hours. After full separation, the gel was submerged in 0.25% Stains All in 50% ethanol on an orbital shaker overnight. The gel was then destained slowly with 10% ethanol.

HA bands in the gel were scanned and quantified using the densitometry software mentioned in section 2.3.

2.7 CYTOKINES

The panel of CD related cytokines used were all obtained from Sigma. The cytokines and their concentrations used were as follows: TNF-α 5ng/mL, IL1-β 0.1ng/mL, IL-6 20ng/mL, and IL-8 20ng/mL, all ordered from PeProTech. The established concentrations were derived from previous studies done with these cytokines to stimulate CD characteristics in ND fibroblasts (97).

150,000 ND fibroblasts were plated in 10mm wells in duplicate and were allowed to grow for three days to allow confluence. On the third day, the media was replaced in both wells, and the well designated for stimulation had the cytokine added at the established concentration. The media and cytokines were then replaced every three days until the 10th day, when the cells and ECM were harvested based on the aforementioned procedure.
CHAPTER 3: KIAA1199 PLAYS A SIGNIFICANT ROLE IN HA DEGRADATION IN COLON FIBROBLASTS, AND CD COLON FIBROBLASTS HAVE ELEVATED LEVELS OF CELLULAR AND SECRETED KIAA1199 COMPARED TO ND.

3.1 INTRODUCTION

The incidence of fibrosis in CD is significant for any patient because of its limited treatment options. While there are medical therapies that can control inflammation, no therapy effectively controls fibrosis in CD patients. Surgical intervention is the most common treatment for intestinal fibrosis stemming from CD.

As described before, fibrosis is characterized by an increase in ECM deposition by activated fibroblasts. One of the major ECM components elevated during fibrosis is the glycosaminoglycan HA. When HA is degraded, the resulting HA fragments have been well documented to be pro-inflammatory and pro-fibrotic (82). There are still many unanswered questions about how proinflammatory HA fragments are created in vivo.

A recent publication has linked the novel protein KIAA1199 to HA degradation (92). KIAA1199’s role in HA fragmentation is significant because HA fragments are well known pro-inflammatory molecules (82), which is important in the progression of CD. This led us to the underlying hypothesis that KIAA1199 is altered in CD colon fibroblasts compared to ND colon fibroblasts and consequently affects HA degradation in CD cells. In the following study, we investigate this novel protein by
examining its expression in CD colon fibroblasts and ND colon fibroblasts along with its function in those cells.

3.2 RESULTS

3.2.1 CD Colon Fibroblasts have Higher Cellular and Secreted Levels of KIAA1199 Compared to ND Colon Fibroblasts

We hypothesized that the levels of KIAA1199 would be altered in CD colon fibroblasts when compared to ND. We measured levels of this protein in the three different compartments of cell culture: whole cell, ECM, and conditioned media. Colon fibroblasts were grown for 3 days and 13 days individually. 3 days time allows the cells to become confluent in their growth plate. 13 days of growth result in the cells depositing high amounts of ECM.

The ECM was studied with a proteomics approach. ECM synthesized from CD and ND colon fibroblasts grown for 13 days were analyzed by mass spectrometry for protein identification. The resulting spectral counts indicate that KIAA1199 protein was increased in the ECM of CD colon fibroblasts compared to the ECM of ND colon fibroblasts (Figure 3-1).

Whole cell lysate isolates of CD and ND colon fibroblasts were measured via immunoblot. When comparing the isolated cellular fractions, levels of KIAA1199 were higher in the CD cells at both 3 days and 13 days of growth (Figure 3-2).
Cultured media from CD and ND colon fibroblasts were also measured via immunoblot. The amount of KIAA1199 secreted by CD fibroblasts into the media at 3 days of growth is higher than KIAA1199 secreted by ND fibroblasts. However, at 13 days of growth, the amount of KIAA1199 secreted into the media is even between CD and ND fibroblasts (Figure 3-3).

3.2.2 CD and ND Fibroblasts Have Similar mRNA Levels of KIAA1199

In investigating causes for the difference in KIAA1199 protein between CD and ND at the cellular level, we asked whether the gene expression level of KIAA1199 differed. Since increased protein level suggests an increase in message, we hypothesized that the CD colon fibroblasts would have elevated KIAA1199 mRNA when compared to ND colon fibroblasts.

At both 3 days of growth and 13 days of growth, qPCR results of the same fibroblast lines used in the previous experiments showed no difference in KIAA1199 mRNA levels (Figure 3-4). This indicates that the CD cells are not inherently making more KIAA1199, but something occurs after translation of the protein that causes the levels to be greater in CD cells than in ND cells.

3.2.3 Fibrotic Colon Tissue of CD Patients appears to have Elevated KIAA1199 in the Muscularis Mucosa compared to the Muscularis Mucosa of a non-IBD Patient

Though only representative of a single fibrotic human colon, detection of KIAA1199 by use of fluorescence histochemistry depicts an elevated level of KIAA1199 in the
muscularis mucosae compared to colon sections from a non-IBD patient (Figure 3-5). The accompanying H&E stained image displays the fibrotic characteristics in the CD colon, where the muscularis mucosae is highly expanded compared to the non-IBD colon. It is important to note that KIAA1199 levels correlate with the expansion of the cells in the muscularis mucosae, but are not elevated in the mucosa of CD compared to the non-IBD mucosa.

3.2.4 KIAA1199 is Visually Depicted as a Cytosolic and Nuclear Associated Protein

KIAA1199 has been identified in numerous locations within specific cancer cells, including in the endoplasmic reticulum (87) and the cellular membrane (90). We wanted to investigate the localization of KIAA1199 in colon fibroblasts of both CD and ND. To obtain a visual representation of KIAA1199 in colon fibroblasts, immunohistochemistry was utilized based on the aforementioned procedure. Both three and 13 day grown fibroblasts were stained for KIAA1199.

The images shown depict KIAA1199 as a cytosolic and nuclear associated protein in all colon fibroblasts (Figure 3-6). However, we observe a unique streaking pattern of KIAA1199 that exists exclusively in the CD colon fibroblasts grown for 13 days (Figure 3-6A Lower Right Panel). This streaking pattern appears to be associated with the matrix rather than cell-associated. In the higher magnification confocal image of 3-day ND fibroblasts (Figure 3-6B), KIAA1199 signal can be seen surrounding the nuclei, indicating affiliation with the nuclei.
3.2.5 KIAA1199 Plays an Integral Role in HA Degradation in Colon Fibroblasts

With previous links to HA degradation in embryonic skin fibroblasts (92), we hypothesized that KIAA1199 plays a role in HA degradation by colon fibroblasts.

Our HA degradation assay measured HA degradation capability of both CD and ND colon fibroblasts normally and with KIAA1199 and HYAL2 silenced individually, with siRNA transfection specifically detailed in Chapter 2 (2.6). Pure, synthetic HA of 1m Da molecular weight was incubated with these transfected fibroblasts. Figure 3-7 shows a representative image of the resulting HA sizing agarose gel which separated HA in the media following incubation with the transfected cells, with proof of silencing via immunoblot represented below the image.

The results indicate that mock transfection cells are capable of reducing HA size, whereas there is a complete loss of HA degradation activity when KIAA1199 is knocked down in both CD and ND colon fibroblasts. When HYAL2 is knocked down, HA degradation activity is also significantly decreased, but not to the extent of KIAA1199 knockdown. Western blot analysis confirmed that KIAA1199 and HYAL2 protein were completely knocked down (Figure 3-7B). Figure 3-7C shows average band quantification from 5 different cell lines, normalized to HA content.

Unexpectedly, KIAA1199 knockdown in cells resulted in the secretion of a high molecular weight band of HA (Figure 3-7D), near 6m Da. This band does not appear when HYAL2 is knocked down, or when cells are treated with siRNA without
electroporation. Additionally, this band is secreted regardless of whether exogenous HA is added or not (not shown). The band was confirmed to be HA by its sensitivity to the HA specific enzyme *S. hyalurolyticus* hyaluronidase (not shown).

3.2.6 KIAA1199 and HYAL2 do not Co-Localize in Colon Fibroblasts

To determine whether KIAA1199 and HYAL2 are located together in cells, fluorescence immunohistochemistry with specific KIAA1199 and HYAL2 antibodies was used to identify the proteins in colon fibroblasts. The resulting confocal image of a non-IBD fibroblast line grown for three days (*Figure 3-8*) suggests that KIAA1199 does not co-localize with HYAL2 in those cells. Additionally, HYAL2 appears to be located in a unique reticular pattern on the cell membrane whereas KIAA1199 is intracellular.

3.2.7 HYAL2 Knockdown Decreases Cellular and Secreted Levels of KIAA1199

*Figure 3-7* indicates that knockdown of KIAA1199 and knockdown of HYAL2 individually decrease HA degradation. This raised the question about how the cell would react to losing a protein involved in HA degradation. We hypothesized that knockdown of either protein upregulates the other; if KIAA1199 is silenced, the cells will make more HYAL2, and if HYAL2 is silenced, the cells will produce more KIAA1199.

Our results show that knockdown of HYAL2 decreases cellular levels of KIAA1199 protein (*Figure 3-9A*). Immunoblots of cell lysate show a downregulation of
KIAA1199 when HYAL2 is knocked down in colon fibroblasts, compared to KIAA1199 levels in cells treated with mock siRNA. The result was consistent over four experiments, as shown in the graph of the blot quantification (Figure 3-9B). This finding is consistent between CD and ND fibroblasts. When measuring KIAA1199 protein in conditioned media of HYAL2 knockdown colon fibroblasts (data not shown), the results are similar to the cellular KIAA1199 levels measured. Thus, the knockdown of HYAL2 in colon fibroblasts results in the decrease of cellular and secreted KIAA1199 protein.

A representative immunoblot (Figure 3-9C) indicates that knockdown of KIAA1199 does not affect cellular HYAL2 levels. Four different samples, both CD and ND, showed this was a consistent finding (Figure 3-9D).

3.3 DISCUSSION

This data, taken together, suggest a role for KIAA1199 protein in HA degradation activity that was upregulated in CD cells. With CD fibroblasts inherently having elevated cellular and matrix levels of KIAA1199 \textit{in vitro} and \textit{in vivo}, (Figure 3-1, Figure 3-2, Figure 3-5) these cells seem likely to be more efficient in degrading HA. However, we think that the function of KIAA1199 may not be direct to HA degradation, but rather may be an HA binding activity that is part of the mechanism of HA degradation by HYAL2.
One surprising result of KIAA1199 silencing, fibroblasts released a specific band of high molecular weight HA (~6m Da). This band was present in carbohydrate gels of media samples from both CD and ND KIAA1199 knockdown fibroblasts, and was present whether or not exogenous HA was added (Figure 3-7). Questions arise as to why there is such a specific size of HA being released when KIAA1199 is knocked down. Alternatively to the hypothesis that KIAA1199 serves to directly degrade HA, it is possible that KIAA1199 is an integral part of the HA degrading pathway that binds matrix associated HA and holds it to the cell. The increased levels of KIAA1199 in CD fibroblasts could be a mechanism that fosters the increased matrix deposition and HA degradation by CD fibroblasts as part of the inflammatory response of CD. As Figure 3-7 shows, without KIAA1199, the cell is releasing a large molecular weight HA that otherwise would be adherent to the cells, while not being able to degrade exogenous HA due to the fact that KIAA1199 plays a role in binding the exogenous HA to the cell. Though we do know that the large molecular weight band in Figure 3-7D is HA, investigating the band further could provide more specificity about the function of KIAA1199. In all, it suggests that KIAA1199 has a role other than HA degradation.

Though other studies (92) and our immunohistochemistry data have suggested that KIAA1199 is not associated with HYAL2, Figure 3-9 indicates that there is some sort of relationship between the two proteins. The immunoblot results imply that HYAL2 is a regulator of KIAA1199 in some facet. It could be that KIAA1199 protein is processed by a pathway that requires HYAL2, or that HYAL2 is genetic
upregulator of KIAA1199. It is also possible that HYAL2 is an inhibitor of a KIAA1199 downregulator. Further investigation into the reactions of colon fibroblasts when HYAL2 is knocked down will help determine other roles of that protein. Additionally, further examination of the relationship between HYAL2 and KIAA1199 is important to determine whether they in fact do work together. Results would have the potential of opening a new door for investigation into KIAA1199-HYAL2 dependent functions in cells, since no information has been reported regarding this association.

The different levels of KIAA1199 protein in the cell and secreted into the conditioned media between CD and ND fibroblasts could point to a malfunction in the metabolism or processing of KIAA1199 within the cell. It would seem that without an increase in mRNA (Figure 3-4), CD cells have increased levels of KIAA1199 protein because they are not properly degrading it intracellularly, or moving it to an extracellular compartment that prevents breakdown. Additionally, the difference in media KIAA1199 protein at 3 days of growth suggests that CD cells are secreting this protein because they are not properly processing it intracellularly. A proteasome assay treatment could be utilized to determine if an intracellular degradation malfunction is the issue in CD cells. This would involve treating ND colon fibroblasts with a proteasome inhibitor, then measuring KIAA1199 in the different cell culture compartments. Repeating this experiment with CD colon fibroblasts and measuring the KIAA1199 protein level changes could perhaps suggest the cause of inherent KIAA1199 protein elevation in CD colon fibroblasts.
At 13 days, both CD and ND fibroblasts have elevated KIAA1199 mRNA (Figure 3-4). In ND fibroblasts, this coincides with an increase in media levels of KIAA1199 protein (Figure 3-3), suggesting a processing pathway for KIAA1199. As previously shown, CD fibroblasts at 3 days already have elevated media and cellular levels of KIAA1199 protein. After the following 10 days, both remain elevated, but with the increase in matrix KIAA1199 protein the significant difference. This further suggests that CD cells preferentially deposit KIAA1199 protein in their ECM. Further experiments could include a time course measurement of KIAA1199 in the cells and matrix to see if the deposition of KIAA1199 occurs gradually, or quickly. This could further provide evidence on the function of KIAA1199 in CD.
Figure 3-1: KIAA1199 Protein is Elevated in CD ECM Compared to ND ECM

A dot plot representing the spectral counts of KIAA1199, data obtained from mass spectrometry analysis of isolated ECM from CD and ND colon fibroblasts grown for 13 days. In total, 3 separate CD cell lines and 2 ND cell lines were measured via mass spectrometry. Results show elevated spectral counts of KIAA1199 in the CD ECM.
Figure 3-2: Intracellular Levels of KIAA1199 are Elevated in CD Colon Fibroblasts Compared to ND Colon Fibroblasts

A

**Figure 3-2 A:** Representative immunoblot images of KIAA1199 levels in whole cell lysate. GAPDH measurement is included as a loading control. A total of 4 ND fibroblast lines and 4 CD fibroblast lines were measured in total. The immunoblot images were simply best representatives of the trend.
Figure 3-2 (cont.)

B

A bar graph representing the average of quantitative densitometry measurements of all immunoblots measuring relative KIAA1199 protein expression in colon fibroblast whole cell lysate. A total of 4 ND cell lines were measured along with 4 CD cell lines. At both 3 and 13 days of growth, intracellular KIAA1199 protein is generally elevated in CD colon fibroblasts compared to ND colon fibroblasts.
Figure 3-3: CD Colon Fibroblasts Secrete Elevated KIAA1199 Compared to ND after 3 Days of Growth, but CD and ND Secrete Similar Levels of KIAA1199 after 13 Days of Growth

A

Figure 3-3 A: Representative immunoblot images of KIAA1199 protein in the media of 3 day and 13 day colon fibroblasts, both ND and CD. For this experiment, two gels were run simultaneously and transferred to the same membrane. The resulting images represent both growth times at the same exposure to film. In other words, in these images, 3 day and 13 day can be compared to one another, along with CD and ND within each growth time.
Figure 3-3 (cont.)

B: A graph depicting the average of the quantitative densitometry analysis of immunoblots measuring KIAA1199 protein level in media cultured by ND and CD colon fibroblasts. A total of 4 ND cell lines and 5 CD cell lines were compared. Results indicated that CD colon fibroblasts secrete more KIAA1199 protein than ND colon fibroblasts after 3 days. However, over the following 10 days, CD and ND colon fibroblasts secrete similar levels of KIAA1199 into the media.
Figure 3-4: mRNA Levels of KIAA1199 at an Equal Level Comparing ND and CD Fibroblasts at 3 Days of Growth and 13 Days of Growth

Figure 3-4 A graph representing average relative KIAA1199 mRNA levels in colon fibroblasts grown for 3 days and 13 days, quantified by qPCR. The data is representative of measurements from 5 ND cell lines and 5 CD cell lines. All individual measurements are compared to the 18S measurement, then to the average of the 3 Day ND relative KIAA1199 mRNA level, in order to determine the change from “normal.” The data indicates that KIAA1199 mRNA is equal between CD and ND fibroblasts at each growth time, but the 13-day grown cells have higher levels of KIAA1199 mRNA compared to 3 day.
Figure 3-5: KIAA1199 Signal is Increased in the Muscularis Mucosae of a Fibrotic Human Colon Compared to a Non-IBD Colon

Figure 3-5 Fluorescent micrographs of a colon tissue section of a fibrotic CD and a non-IBD colon tissue, accompanied by a micrograph of the same tissue section in the same area with H&E staining. The yellow arrows point to the muscularis mucosae layer of the tissue section. In the fluorescent micrograph, KIAA1199 is stained in red, and the nuclei are stained in blue with DAPI.
Figure 3-6: Fluorescent Staining Localizes KIAA1199 to the Cytoplasm and Nucleus

A

Figure 3-6 A: Fluorescent micrographs of ND and CD colon fibroblasts grown for 3 and 13 days. KIAA1199 is fluorescently immunolabeled in red, while the nuclei are stained with DAPI (blue). These images depict KIAA1199 as a cytosolic protein and 13-day grown CD fibroblasts appear to have signal in the extracellular portion of the cells.
Figure 3-6 (cont.)

B

Figure 3-6 (cont.) B: A confocal micrograph of ND colon fibroblasts grown for 3 days. KIAA1199 is fluorescently immunolabeled in red, and the nuclei are labeled with DAPI (blue). This image visually depicts KIAA1199 surrounding the nuclei of the fibroblasts, along with its presence in the cytoplasm.
Figure 3-7: KIAA1199 Plays an Integral Role in Exogenous HA Degradation in Colon Fibroblasts

A

Figure 3-7 A: A representative scan of an HA sizing agarose gel depicting the results of the HA Degradation Assay. The bands in the image depict 1m Da HA after incubation in one of the following conditions: No cells, colon fibroblasts electroporated with scramble siRNA (mock), colon fibroblasts electroporated with KIAA1199 siRNA, and colon fibroblasts electroporated with HYAL2 siRNA. Lower strength of the band indicates that the added HA has been degraded. B: Immunoblot results confirming either the silencing or non-silencing of the intended protein, with GAPDH to compare. All cells were treated via electroporation.
Figure 3-7 (cont.) C:

Graph depicting densitometry quantification of average fold change in exogenous HA after incubation, representing all HADAs run, 5 cell lines in total (3 CD and 2 ND). Going along with the image in 3-7A, KIAA1199 knockdown in colon fibroblasts results in a diminished ability to degrade exogenous HA.

Furthermore, knockdown of HYAL2 in colon fibroblasts also abrogates HA degradation, but not to the same extent of KIAA1199 knockdown. Comparatively, cells electroporated with mock siRNA are still able to degrade exogenous HA.
Figure 3-7 (cont.)

D

Figure 3-7 (cont.) D: Expansion of the image from 3-7A showing high molecular weight (~6m Da) band of HA exclusively in the KIAA1199 knockdown media sample. Seen in all HADAs done, this band was confirmed to be HA (data not shown) and is secreted exclusively when colon fibroblasts are electroporated with KIAA1199 siRNA. Thus, the KIAA1199 in the cell does not allow this high molecular weight band of HA to be secreted.
Figure 3-8: KIAA1199 and HYAL2 do not Co-Localize in Colon Fibroblasts

Figure 3-8 A confocal micrograph of ND colon fibroblasts grown for 3 days. Both HYAL2 (green) and KIAA1199 (red) are fluorescently immunolabeled. The image depicts HYAL2 as a membrane protein, branched out in a unique pattern, while KIAA1199 is localized to the cytoplasm and nucleus. The proteins do not appear to co-localize. The nuclei of the cells are stained with DAPI (blue).
Figure 3-9: Knockdown of HYAL2 in Colon Fibroblasts Results in Decrease of Cellular Levels of KIAA1199 Protein

A

**Figure 3-9 A**: Representative immunoblot depicting KIAA1199 protein levels measured in the whole cell lysate of HYAL2 knockdown colon fibroblasts and scramble siRNA (mock) colon fibroblasts. Colon fibroblasts with silenced HYAL2 expression downregulate KIAA1199 protein.
Figure 3-9 (cont.)

B

Figure 3-9 (cont.) B: Graph representing the averages of densitometric quantification of immunoblot bands from measurements of KIAA1199 levels in whole cell lysates of HYAL2 knockdown cells and scramble (mock) siRNA cells. These results average four separate samples (2 CD and 2 ND) measured by immunoblot, compared to GAPDH for normalization. The results confirm the trend depicted in 3-9A, that HYAL2 knockdown colon fibroblasts downregulate cellular KIAA1199 protein.
Figure 3-9 (cont.)

C:

Figure 3-9 (cont.): Representative immunoblot images showing HYAL2 levels in the whole cell lysate of KIAA1199 knockdown colon fibroblasts and scramble (mock) siRNA colon fibroblasts. HYAL2 protein levels are not affected by the knockdown of KIAA1199 in colon fibroblasts.
Figure 3-9 (cont.)

D

**HYAL2 Protein Levels in KIAA1199 Knockdown Colon Fb**

<table>
<thead>
<tr>
<th>Relative Protein Expression (HYAL2/GAPDH)</th>
<th>Scramble siRNA</th>
<th>KIAA1199 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
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<td>0.5</td>
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</tbody>
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**Figure 3-9 (cont.)** D: Graph representing the averages of densitometric quantification of immunoblot bands from measurements of HYAL2 protein levels in whole cell lysates of KIAA1199 knockdown cells and scramble (mock) siRNA cells. These results average four separate samples (2 CD and 2 ND) measured by immunoblot, compared to GAPDH for normalization. The results confirm the trend depicted in **3-9C**, that KIAA1199 knockdown does not affect HYAL2 protein levels in colon fibroblasts.
CHAPTER 4: KIAA1199 IN COLON FIBROBLASTS IS UPREGULATED TO THE ECM BY IL-6 STIMULATION.

4.1 INTRODUCTION

The cytokines involved in an inflammatory response are of numerous. These cytokines can be produced by multiple cell types activated by a variety of stimuli. Each of these cytokines trigger a pathway that upregulates more cytokines and other pro-inflammatory molecules that contribute to the inflammatory response. In all, investigating cytokines stimulation is an important path to take when examining at CD pathology.

In investigating the role of KIAA1199 in CD, looking at how and if cytokines regulate the protein's level and localization could link it to a certain pathway in a CD related inflammatory response. For the subsequent experiments, the following four cytokines were used: tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL1-β), interleukin 6 (IL-6), and interleukin 8 (IL-8). These cytokines were chosen because they are highly expressed in IBD intestine during inflammation.

TNF-α is a pro-inflammatory cytokine produced by a variety of cell types, predominantly macrophages (5). TNF-α is a stimulator of NF-kβ and induces activation of the Januse kinase (Jnk) pathway, both of which result in an inflammatory response in a variety of ways (98). Studies have indicated that HA fragments induce production of TNF-α (82, 99). Furthermore, there is evidence of
elevated TNF-α levels in CD patients’ stool (100). Anti-TNF-α therapy is a commonly used as a treatment for CD (5) to attempt to alleviate the inflammation.

IL1-β is a pro-inflammatory cytokine that is involved in proliferation and angiogenesis along with the inflammatory response. Notably, it influences molecules that contribute to the physiological response to inflammation. This cytokine induces the synthesis of numerous inflammatory and adhesion molecules via leukocyte activation (101). It is produced primarily by activated macrophages and can be induced by HA fragments (102, 103). IL-1β has also been implicated in CD, with studies having found elevated levels in the colonic mucosa of CD patients (104).

IL-6 is a cytokine and a myokine that affects a wide range of cells. Interestingly, it has both pro-inflammatory and anti-inflammatory functions. As a myokine, IL-6 is released from muscle cells during physical exercise and then mediates anti-inflammatory effects in circulation (105, 106). As a pro-inflammatory cytokine, IL-6 itself can stimulate growth and differentiation of immune cells, notably T-cells and B-cells (109). IL-6 induction can occur via inflammatory cytokine or HA fragment activation of leukocytes (107, 108), among other mechanisms. In CD, IL-6 was found elevated in the serum of CD patients compared to ND patients (113). In addition, anti-IL-6 therapy is a new approach to treatment of inflammation in CD (114). More studies need to be done on IL-6 to understand the seemingly contrasting roles it plays in regards to inflammation.
IL8 is a chemokine involved in angiogenesis and leukocyte recruitment (107). IL-8 can act in numerous inflammatory processes, notably by stimulating neutrophil activity (115). IL-8 production can be stimulated in a variety of inflammatory cells by interactions with other inflammatory cytokines and HA fragments (108, 115).

The data previously presented (Chapter 3) indicates that KIAA1199 is elevated in CD colon fibroblasts and furthermore has a role in the inflammatory relevant process of HA degradation. All of the aforementioned cytokines can be induced via HA fragment signaling. With this, we hypothesized that ND cells stimulated by CD related cytokines will upregulate KIAA1199 protein, indicating the protein's role in the inflammatory response via HA degradation.

4.2 RESULTS

4.2.1 IL-6 elevates KIAA1199 in the ECM of Stimulated ND Colon Fibroblasts

Cytokines drive inflammation in a variety of ways. With the previous study, we determined that KIAA1199 is involved in production of inflammatory stimulating HA fragments by colon fibroblasts. With the elevated levels in CD cells, there is the question whether KIAA1199 elevation is part of the inflammatory response resulting from cytokine stimulation.

We measured cellular and ECM KIAA1199 protein levels in stimulated and unstimulated ND fibroblasts. Our data revealed that KIAA1199 protein is
upregulated in the matrix by IL-6. There is a possibility that TNF-α is also an upregulator of KIAA1199, but a repeated experiment did not see the same trend.

We did not find any significant differences in cellular KIAA1199 when stimulated with these cytokines (Figure 4-2). The graph depicts IL-6 as a downregulator of cellular KIAA1199, but this trend was not reproducible, so the result was likely an outlier.

4.3 DISCUSSION

The upregulation of KIAA1199 in the ECM is essentially the basis of this project. That upregulation was detected exclusively in CD matrix. The data presented here suggests that during CD, it is the stimulation by IL-6 that is the specific cause of the KIAA1199 deposition. That could implicate a variety of inflammatory processes to KIAA1199, for multiple types of immune stimulation produce IL-6. Additionally, IL-6 induces the production of numerous types of inflammatory cytokines through different pathways (112). The exogenously added IL-6 could be directly stimulating KIAA1199 deposition in the matrix, or could be inducing the synthesis of another inflammatory factor that increases KIAA1199 in the ECM. This preliminary experiment provides the framework for more specific work investigating IL-6’s relationship with KIAA1199.

Further experiments would be needed to measure both how IL-6 is stimulating KIAA1199 deposition in the ECM and the consequence of this increase of KIAA1199
in matrix. Treatment of IL-6 receptor blocked cells with conditioned supernatant from IL-6 stimulated colon fibroblasts would help determine whether IL-6 acts in a direct or indirect manner to upregulate KIAA1199 deposition in the ECM. Experiments measuring levels of different inflammatory factors following IL-6 treatment could link KIAA1199 to other inflammatory factors, or rule some cytokines out. Additional stimulation experiments with further cytokines, especially fibrogenic cytokines, could link KIAA1199 to further inflammatory and fibrotic pathways. Measuring the HA degradation activity of IL-6 stimulated colon fibroblasts compared to unstimulated fibroblasts would indicate whether the IL-6 stimulation affects HA degradation capability.

The immunoblot results would make it seem that TNF-α upregulates KIAA1199 in the ECM as well, but this trend was not reproducible. It is very likely there was a mechanical issue protein measurement or loading. The decreased level of KIAA1199 in the whole cell lysate of IL-6 stimulated cells also appears to be an outlier or a mechanical issue with the experiment.
Figure 4-1: IL-6 Upregulates KIAA1199 Protein in the ECM of non-IBD Colon Fibroblasts

A

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<td>IL-1β</td>
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<td>IL-8</td>
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**Figure 4-1 A:** Representative immunoblot of KIAA1199 protein in extracellular matrix isolates from non-IBD colon fibroblasts grown for 13 days, either untreated or treated with the indicated cytokine every three days following confluence. Please note that the change in TNF-α stimulated fibroblasts was **not** reproduced in a second experiment. The change in IL-6 was reproduced.
**Figure 4-1 (cont.)**

B

**Cytokine Induced Changes of KIAA1199 Protein Levels in Colon Fb: Extracellular Matrix**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Unstimulated</th>
<th>Stimulated</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>12,000,000</td>
<td>14,000,000</td>
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<tr>
<td>IL-1β</td>
<td>8,000,000</td>
<td>10,000,000</td>
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<tr>
<td>IL-6</td>
<td>16,000,000</td>
<td>18,000,000</td>
</tr>
<tr>
<td>IL-8</td>
<td>6,000,000</td>
<td>8,000,000</td>
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**Figure 4-1 (cont.)** B: Densitometric quantification of the immunoblot measuring differences in KIAA1199 protein levels in the ECM of stimulated vs. unstimulated cells by the indicated cytokines. Densitometry of the immunoblots indicate the IL-6 increases KIAA1199 levels in the ECM of ND fibroblasts.
Figure 4-2: Selected CD Related Inflammatory Cytokines do not Induce a Change in KIAA1199 Protein Levels in non-IBD Colon Fibroblasts

A

Figure 4-2 A: Representative immunoblot image of KIAA1199 levels relative to GAPDH in the whole cell lysate of stimulated and unstimulated ND colon fibroblasts.

All samples were grown for 3 days, then untreated for 10 days or treated with cytokines every three days for 10 more days.
Figure 4-2 (cont.)

B

**KIAA1199 Protein in Cytokine Stimulated ND Colon Fb: Whole Cell Lysate**

Figure 4-2 (cont.) B: Representative graph of the averages of densitometric quantifications of KIAA1199 protein levels relative to GAPDH in the whole cell lysates of stimulated ND colon fibroblasts. All samples were grown for 3 days, then untreated for 10 days or treated with cytokines every three days for 10 more days. This graph represents two separate experiments with 2 different ND cell lines. Please note that the change in IL-6 was **not** reproduced.
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTION

5.1 SUMMARY OF FINDINGS

KIAA1199 protein is elevated in CD colon fibroblasts along with increased deposition into the ECM of those cells. This occurs without an increase in message, as ND and CD cells have equal KIAA1199 mRNA levels. KIAA1199 appears to be a nuclear and cytosolic protein in colon fibroblasts. KIAA1199 plays an integral role in HA degradation, so much that colon fibroblasts without KIAA1199 cannot degrade exogenous HA. In addition, KIAA1199 knockdown colon fibroblasts release a high molecular weight HA unique to that condition. It was discovered that knocking down HYAL2 in colon fibroblasts downregulates intracellular and secreted KIAA1199. Knocking down KIAA1199 does not affect HYAL2 levels in colon fibroblasts. KIAA1199 seems to be upregulated in the matrix of colon fibroblasts by stimulation of IL-6.

5.2 CONCLUSIONS

This investigation into KIAA1199 gives many clues to its role in CD while subsequently opening the door to many more questions about its normal function. While we understand very little about this protein, the information we have obtained has been a certain step forward in discovering it’s role in HA fragment production, which will surely lead to further data on how it can effect CD patients at the cellular level.
Primarily, expression studies of KIAA1199 protein indicating its increase in CD colon fibroblasts depict another possible malfunction in CD with its resulting consequences. The elevated deposition of KIAA1199 into the ECM following stimulation by IL-6 further relates KIAA1199 to inflammation in CD. Its function in HA degradation is not completely known, but the fact that it plays an integral role in this process in colon fibroblasts means it has potential to be a significant player in the inflammatory and fibrotic conditions in the colon of CD patients.

5.3 FUTURE DIRECTION

5.3.1 KIAA1199 and Hyaluronan

Our data makes the clear link between KIAA1199 and HA. Simply put, without KIAA1199, colon fibroblasts cannot degrade exogenous HA. In addition, the loss of KIAA1199 causes the secretion of high molecular weight HA from the cell (Figure 3-7). This gives evidence of a possible role in HA-binding along with HA degradation, either in the cell or on the matrix.

The beneficial presence of high molecular weight HA in the ECM has been well studied (45). Additionally, a recent study gave evidence that suggested that KIAA1199 has HA-specific binding capabilities (92). While our data has shown KIAA1199 as a cytosolic and ECM protein, further studies need to be done to depict in what capacity KIAA1199 is binding HA.
It would be important to determine whether the high molecular weight band released following knockdown of KIAA1199 is related in any way to the abrogation of HA degradation in the same condition. On one hand, it would make sense that the high molecular weight band of HA released by the cell is the HA created by the cell and not catabolized properly due to the lack of KIAA1199. Some questions in that regard stem from the fact that the cell is releasing a specific size of ~6m Da. On the other hand, the presence of cytosolic KIAA1199 could depict an intracellular role of HA binding. The finding that KIAA1199 protein is elevated in CD colon fibroblasts suggests a role of KIAA1199 in CD cells. If KIAA1199 is binding intracellular HA, that could indicate a role of intracellular HA in CD. This theory has merit, for there have been findings of intracellular HA and HA-binding proteins, and there are suggestions that intracellular HA could play a role in inflammation (59, 110).

Histochemical analyses need to be done to determine the relationship between KIAA1199 and HA. Co-localization of the two inside or on the exterior of colon fibroblasts would suggest an interaction. From there, further studies could be done to determine the purpose of a KIAA1199-HA interaction. Knocking down KIAA1199 followed by staining for HA could give light on how the structure of intracellular or extracellular HA changes with the absence of HA.

An alternative method of investigation into the consequences of KIAA1199 silencing could be to directly analyze the sharp band of high molecular weight HA that was secreted. It is entirely possible that the high molecular weight HA is part of an
aggregate, a combination of HA and hyaldherins that is released from the cell. A mass spectrometry analysis of the specific band could identify the proteins, if any, which are being released with that large HA. In turn, any results could provide a further direction to investigate: KIAA1199 is related to a specific hyaldherin that is found with that released HA, or KIAA1199 is itself a hyaldherin or has some other responsibility to keep that high molecular weight HA with the cell.

In either case, the framework is set for further investigation into a direct relationship between KIAA1199 and HA.

5.3.2 KIAA1199 and HYAL2

A relationship between KIAA1199 and HYAL2 was initially not perceived based on the data from the prior group studying KIAA1199 and HA degradation. They showed that knocking down HYAL2 did not abrogate HA degradation in embryonic skin fibroblasts, suggesting that KIAA1199 is an independent HA degradation protein (92). However, we have data that suggests there is a relationship between the two HA related proteins.

Primarily, in our colon fibroblasts, knocking down HYAL2 significantly decreased exogenous HA degradation (Figure 3-7). Furthermore, the same data shows a virtually complete abrogation of exogenous HA degradation by colon fibroblasts without KIAA1199. Therefore, neither HYAL2 nor KIAA1199 alone is enough to completely degrade HA in colon fibroblasts.
To further investigate this loss of function, we wanted to determine the cell's reaction to the loss of one protein. Our results indicated that the loss of HYAL2 coincided with the significant decrease of cellular and secreted KIAA1199 (Figure 3-9). The loss of KIAA1199 did not affect the cellular levels of HYAL2. These data suggest that HYAL2 is involved in the regulation of KIAA1199. One explanation could be that HYAL2 is part of a pathway that upregulates KIAA1199 production, or that it is a part of a pathway that downregulates a KIAA1199 inhibitor. This is not unforeseen, since HYAL2 has been indicated as a regulatory factor in other instances (111).

To further investigate this relationship, we want to look deeper into the consequences of knockdown of either of the proteins. We could use qPCR to find out if levels of KIAA1199 mRNA levels change with the knockdown of HYAL2, which would provide insight on the regulatory nature of HYAL2 in regards to KIAA1199. Additionally, staining for KIAA1199 when HYAL2 is knocked down would provide a visualization of the change in cellular KIAA1199. It would also serve to reveal if there is a change in localization of KIAA1199 that is diminishing HA degradation capability. Conversely, doing an immunohistochemical analysis of HYAL2 in KIAA1199 knockdown fibroblasts could provide evidence that explains why HA degradation is abrogated even with the presence of HYAL2. Our confocal image of ND colon fibroblasts co-stained for KIAA1199 and HYAL2 shows HYAL2 in a specific conformation encompassing the cell membrane (Figure 3-8). A change in that specific conformation would suggest that KIAA1199 is somehow involved in the
membrane localization of HYAL2 which consequently has a direct effect on its HA degradation capability. Immunoprecipitation of KIAA1199 could determine if it creates a complex with HYAL2, which would strengthen the theory that the two proteins work together.

One protein we haven't investigated in this regard is CD44, the well known HA receptor involved in HYAL2 degradation and subsequent internalization (51, 65, 82). Since CD44 is also required for HYAL2 mediated HA degradation (65, 82), it is certainly possible that there is a relationship between CD44 and KIAA1199, either related to HA binding or HA degradation. Doing an HA degradation assay with CD44 silenced colon fibroblasts could provide a new aspect to the HYAL2-KIAA1199 relationship and possibly insight into a KIAA1199-mediated HA degradation relationship with CD44. Determining the cellular CD44 levels following KIAA1199 knockdown and HYAL2 knockdown would indicate whether there is a regulatory relationship between the proteins, as would measuring HYAL2 and KIAA1199 protein levels in CD44 knockdown cells. Furthermore, immunostaining colon fibroblasts for CD44 and KIAA1199 or HYAL2 would provide a visual depiction of the relationship between the proteins.

There are many possibilities with the KIAA1199-HYAL2 relationship. In all, we have reason to further investigate the possibility of an interaction between the two, in regards to HA degradation and possibly other functions. With the addition of CD44
into the equation, it could strengthen the current argument that KIAA1199 is a key player in HA turnover in the human body.
BIBLIOGRAPHY


