2008

Rotor Position and Vibration Control for Aerospace Flywheel Energy Storage Devices and Other Vibration Based Devices

B. X. S. Alexander  
Cleveland State University

Follow this and additional works at: https://engagedscholarship.csuohio.edu/etdarchive

Part of the Electrical and Computer Engineering Commons

How does access to this work benefit you? Let us know!

Recommended Citation
https://engagedscholarship.csuohio.edu/etdarchive/6

This Dissertation is brought to you for free and open access by EngagedScholarship@CSU. It has been accepted for inclusion in ETDArchive by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.
THE ROLE OF PLATELETS IN HYALURONAN DEGRADATION

SAMI ALBEIROTI

Bachelor of Science in Biology
Ohio State University
June 2007

Bachelor of Science in Microbiology
Ohio State University
June 2009

submitted in partial fulfillment of requirements for the degree

DOCTOR OF PHILOSOPHY IN CLINICAL AND BIOANALYTICAL CHEMISTRY

at the

CLEVELAND STATE UNIVERSITY

DECEMBER 2014
We hereby approve this dissertation for

Sami Albeiroti

Candidate for the Doctor of Philosophy in Clinical-Bioanalytical Chemistry degree for the

Department of Chemistry

and the CLEVELAND STATE UNIVERSITY

College of Graduate Studies

Dissertation Co-Chairperson, Dr. Carol de la Motte
Department of Pathobiology, Cleveland Clinic
Date

Dissertation Co-Chairperson, Dr. Yan Xu
Department of Chemistry
Date

Dissertation Committee Member, Dr. Vincent Hascall
Department of Biomedical Engineering, Cleveland Clinic
Date

Dissertation Committee Member, Dr. David Anderson
Department of Chemistry
Date

Dissertation Committee Member, Dr. Aimin Zhou
Department of Chemistry
Date

Date of Defense: December 2, 2014
I dedicate this work to my family.

To my father Nabil, who is my role model and source of inspiration, and my mother Jomana for her endless love, dedication and affection.

To my brother, and best friend, Salem for always believing in and supporting me in every way possible and my sister Alia, whose strength and sacrifice inspire me.

And To my beautiful wife Maryam and our son Zayn, thank you for filling my life with joy.
ACKNOWLEDGEMENT

First and foremost, I would like to acknowledge and express my sincere gratitude to my advisor and mentor, Dr. Carol de la Motte. Without her expertise, knowledge, and advice, this work could not have been made possible. Her exceptional kindness and thoughtfulness were critical in contributing to my success and in shaping my personality.

I also wish to acknowledge and thank the de la Motte lab family for their endless help and support: Dr. Sean Kessler, Dr. Aaron Petrey, Dr. David Hill, Dana Obery, Yeo Jung Kim, and Hyujin Rho. A special thank you to Artin Soroosh for all the brainstorming, and fun, conversations we had during many coffee breaks.

I wish to thank all of the faculty and trainees of the Programs of Excellence in Glycosciences, especially Dr. Mark Lauer and Dr. Tony Calabro for many useful conversations, and Dr. Amina Abbadi for being a great colleague, and friend, throughout this journey.

I also would like to acknowledge Dr. Judith Drazba for assistance with confocal microscopy, Ms. Mei Yin for assistance with electron microscopy, Ms. A. Rocio Lopez for assistance with statistics, Dr. Bo Shen for providing patient samples, Katayoun Ayasoufi for help with flow cytometry, and Ms. Gail West for teaching me everything I need to know about cell culture and also for being a great friend and source of support.

I also wish to thank Dr. Sihe Wang for believing in me and for helping me pursue my career goals, Dustin Bunch for his help during my rotation at the Cleveland Clinic Laboratories, and Dr. Joe El-Khoury, for his endless help and support as a colleague and as a friend throughout my PhD years.
I also would like to thank the administrative staff of both the Department of Pathobiology at Cleveland Clinic, and the Department of Chemistry at Cleveland State University for all their help.

Finally, I would like to acknowledge and thank my committee members for their time and advice: Dr. Yan Xu, Dr. David Anderson, and Dr. Aimin Zhou. A special thank you to Dr. Vincent Hascall, whose deep knowledge, exceptional expertise, and critical reading of the dissertation contributed greatly to this work.
THE ROLE OF PLATELETS IN HYALURONAN DEGRADATION

SAM ALBEIROTI

ABSTRACT

Following tissue injury, platelets rapidly interact with the exposed extracellular matrix (ECM) of blood vessel wall and the surrounding tissues. Hyaluronan (HA) is a major polysaccharide component of the ECM and plays a significant role in regulating inflammation. Human platelets can degrade HA from the surfaces of activated endothelial cells into fragments capable of inducing immune responses by monocytes. In addition, platelets contain the enzyme hyaluronidase-2 (HYAL2), one of two major hyaluronidases that digest HA in somatic tissues. The deposition of HA increases in the inflamed tissues in several inflammatory diseases, including Inflammatory Bowel Disease (IBD). This study provides evidence that IBD patients’ platelets have low HYAL2 levels and activity and defines the mechanism by which platelets degrade HA in the inflamed tissues. Human platelets degrade the pro-inflammatory matrix HA through the activity of HYAL2. Importantly, platelet activation causes the immediate translocation of HYAL2 from a distinct population of α-granules to platelet surfaces, where it exerts its catalytic activity. Additionally, platelet activation causes increased binding of platelets to HA. The study also shows that platelets express the novel HA-binding protein KIAA1199, which they release upon activation. Our data provide a new perspective on how platelets interact with their surrounding tissues and explain a possible novel role for platelets in inflammation.
# TABLE OF CONTENTS

Page

ABSTRACT................................................................................................. vi

LIST OF TABLES......................................................................................... xiii

LIST OF FIGURES....................................................................................... xiv

ABBREVIATIONS ........................................................................................... xvi

CHAPTER

I. BACKGROUND............................................................................................ 1

1.1. The Extracellular Matrix........................................................................ 1

1.1.1 Glycosaminoglycans........................................................................... 2

1.2. Hyaluronan............................................................................................. 3

1.2.1. Hyaluronan Structure...................................................................... 3

1.2.2. Hyaluronan Synthesis..................................................................... 6

1.2.3. Hyaluronan Binding Proteins......................................................... 6

1.2.4. Hyaluronan Catabolism................................................................. 7

1.2.4.1. HYAL1......................................................................................... 8

1.2.4.2. HYAL2......................................................................................... 9
1.2.4.3. HYAL2 as a Virus Receptor ................................. 10
1.2.4.4. A Model for HA Degradation .................................. 10
1.2.4.5. KIAA1199 .......................................................... 13
1.2.4.6. Non-Enzymatic Means of HA Degradation .............. 14
1.2.5. HA Function is Size-Dependent ................................. 15
1.2.6. HA in Inflammation ............................................... 17
  1.2.6.1 Role of HMWHA in Inflammation ......................... 17
  1.2.6.2 Role of LMWHA in Inflammation ......................... 19
  1.2.6.3. Pathological HA Cables ..................................... 20
  1.2.6.4. HA Cables in Intestinal Inflammation ................. 23
1.3. Inflammatory Bowel Disease ....................................... 26
1.4. Platelets ............................................................. 26
  1.4.1. Platelet in Inflammation ...................................... 30
  1.4.2. Platelets in IBD .................................................. 31
  1.4.3. Platelet HYAL2 .................................................... 32

II. PLATELET HYALURONIDASE-2 BECOMES SURFACE-EXPRESSED UPON
ACTIVATION TO FUNCTION IN EXTRACELLULAR MATRIX
DEGRADATION ............................................................ 34
2.1. ABSTRACT…………………………………………………………………….. 34

2.2. INTRODUCTION……………………………………………………………….. 35

2.3. MATERIALS AND METHODS……………………………………………….. 37

2.3.1. Materials……………………………………………………………………. 37

2.3.2. Cell Isolation and Culture………………………………………………….. 38

2.3.3. Platelet Isolation……………………………………………………………. 38

2.3.4. Cell co-culture assay for platelet hyaluronidase activity and fragment generation…………………………………………………………………………………. 38

2.3.5. Platelet microparticle isolation…………………………………………….. 39

2.3.6. Immunohistochemical staining of platelets……………………………… 40

2.3.7. Flow Cytometry……………………………………………………………. 42

2.3.8. Subcellular fractionation of platelets using differential centrifugation…………………………………………………………………………………………. 42

2.3.9. Immuno-electron microscopy…………………………………………….. 42

2.3.10. Statistical analysis…………………………………………………………. 43

2.3.11. Immunofluorescence histochemical staining of M-SMCs……………… 43

2.3.12. HA isolation and sizing……………………………………………………… 44

2.3.13. Hyaluronidase assay using immobilized purified HA………………… 44
2.3.14. Hyaluronidase assay using purified HA in solution.................. 45

2.3.15. Subcellular fractionation of platelets using sucrose density gradient.............................................................. 45

2.4. RESULTS AND DISCUSSION................................................................. 45

2.4.1. Platelets degrade pro-inflammatory matrix HA......................... 45

2.4.2. Degradation of matrix HA by platelets is HYAL2 dependent...... 52

2.4.3. Degradation of purified HA by platelets and platelet lysates only occurs at low pH....................................................... 56

2.4.4. HYAL2 becomes surface-expressed on thrombin receptor-activated platelets......................................................... 60

2.4.5. Platelet HYAL2 is localized to a distinct population of granules.............................................................. 67

2.4.6. Activated platelets have higher HAase activity than non-activated platelets.............................................................. 73

2.4.7. IBD patients have lower platelet HYAL2 protein and HAase activities than non-IBD controls................................. 76

2.5. CONCLUSION.................................................................................... 79

III. THE ROLE OF HYALURONAN BINDING PROTEINS AND PLATELET ACTIVATION IN PLATELET-HYALURONAN BINDING......................... 80
3.1. ABSTRACT........................................................................................................ 80

3.2. INTRODUCTION.............................................................................................. 81

3.3. MATERIALS AND METHODS......................................................................... 83
  3.3.1. Materials........................................................................................................ 83
  3.3.2. Cell isolation and culture............................................................................... 84
  3.3.3. Platelet Isolation............................................................................................ 84
  3.3.4. HA Agarose Gel Electrophoreses............................................................... 85
  3.3.5. Immunohistochemical staining of platelets............................................... 85
  3.3.6 Flow Cytometry.............................................................................................. 86
  3.3.7. Platelet microparticles and releasate isolation........................................... 86

3.4. RESULTS AND DISCUSSION.......................................................................... 87
  3.4.1. Platelets digest HA released from poly:1:C-stimulated M-SMCs, but not purified HA, in solution................................................................. 87
  3.4.2. Platelet activation results in increased platelet-HA binding................. 90
  3.4.3. Platelets express the novel HA-binding protein KIAA1199............. 92
  3.4.4. Platelets express KIAA1199 intracellularly and release it upon activation......................................................................................................................... 95

IV. DISCUSSION, FUTURE DIRECTIONS, AND CONCLUDING REMARKS......... 99
**LIST OF TABLES**

Table | Page
--- | ---

**Table 2.1:** List of antibodies used for the immunohistochemical staining of platelets. 41
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 1.1:</strong> The disaccharide that represents the building block of hyaluronan</td>
<td>5</td>
</tr>
<tr>
<td><strong>Figure 1.2:</strong> Representation of a proposed model for HA degradation in cells</td>
<td>12</td>
</tr>
<tr>
<td><strong>Figure 1.3:</strong> HA cables on the surface of poly:I:C-stimulated mucosal intestinal smooth muscle cells (M-SMCs)</td>
<td>22</td>
</tr>
<tr>
<td><strong>Figure 1.4:</strong> Detection of HA structures within the blood vessels of an inflamed colon of a mouse and a human IBD patient</td>
<td>25</td>
</tr>
<tr>
<td><strong>Figure 1.5:</strong> Transmission electron microscopy of imaged of resting and activated platelets</td>
<td>29</td>
</tr>
<tr>
<td><strong>Figure 2.1:</strong> Platelets digest HA in the inflammatory matrix</td>
<td>48</td>
</tr>
<tr>
<td><strong>Figure 2.2:</strong> Platelets degrade pro-inflammatory matrix HA using HYAL2</td>
<td>54</td>
</tr>
<tr>
<td><strong>Figure 2.3:</strong> Platelets and platelet lysates can only degrade purified HA under acidic pH</td>
<td>57</td>
</tr>
<tr>
<td><strong>Figure 2.4:</strong> HYAL2 becomes surface expressed upon thrombin receptor-mediated platelet activation</td>
<td>62</td>
</tr>
<tr>
<td><strong>Figure 2.5:</strong> HYAL2 is stored in a distinct subset of α-granules in resting platelets</td>
<td>69</td>
</tr>
</tbody>
</table>
Figure 2.6: Thrombin receptor-mediated platelet activation results in increased platelet HAase activity. 74

Figure 2.7: Platelets from IBD patients have lower HYAL2 and HAase activity than their non-IBD counterparts. 77

Figure 3.1: Platelets degrade cell-released HA in solution. 89

Figure 3.2: Activated platelets have higher affinity to HA than resting platelets. 91

Figure 3.3: Platelets express KIAA1199. 93

Figure 3.4: Platelets express KIAA1199 intracellularly. 97

Figure 3.4 (cont.): KIAA1199 becomes part of platelet releasate upon activation. 98

Figure 4.1: Low platelet HYAL2 contributes to IBD pathogenesis. 106
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACD</td>
<td>acid-citrate-dextrose</td>
</tr>
<tr>
<td>APC</td>
<td>allophycoerythrin</td>
</tr>
<tr>
<td>APR</td>
<td>acute phase response</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD44</td>
<td>cluster of differentiation 44</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DS</td>
<td>dermatan sulfate</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
</tr>
<tr>
<td>DTS</td>
<td>dense tubular system</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENTV</td>
<td>enzootic nasal tumor virus</td>
</tr>
<tr>
<td>ENV</td>
<td>envelope protein</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HARE</td>
<td>hepatic hyaluronan clearance receptor</td>
</tr>
<tr>
<td>HAS</td>
<td>hyaluronan synthase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ BSS</td>
</tr>
<tr>
<td>HMWHA</td>
<td>high molecular weight hyaluronan</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>HYAL</td>
<td>hyaluronidase</td>
</tr>
<tr>
<td>HβD2</td>
<td>human β-defensin 2</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IαI</td>
<td>inter-α-trypsin inhibitor</td>
</tr>
<tr>
<td>JSRV</td>
<td>Jaagsiekte sheep retrovirus</td>
</tr>
<tr>
<td>KS</td>
<td>keratan sulfate</td>
</tr>
<tr>
<td>LAMP</td>
<td>lysosomal-associated membrane protein</td>
</tr>
<tr>
<td>LMWHA</td>
<td>low molecular weight hyaluronan</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LYVE-1</td>
<td>lymphatic vessel endothelial hyaluronan receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-associated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>M-SMCs</td>
<td>mucosal smooth muscle cells</td>
</tr>
<tr>
<td>MUC5B</td>
<td>mucin 5B</td>
</tr>
<tr>
<td>NEU1</td>
<td>neuroaminidase 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>NHE1</td>
<td>Na(^{+})-H(^{+}) exchanger</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>PGE1</td>
<td>prostaglandin E1</td>
</tr>
<tr>
<td>Poly:I:C</td>
<td>polyinosinic acid:polycytidylic acid</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet-rich plasma</td>
</tr>
<tr>
<td>RHAMM</td>
<td>receptor for hyaluronan-mediated motility</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SPAM1</td>
<td>sperm adhesion molecule</td>
</tr>
<tr>
<td>TGF-(\beta)1</td>
<td>transforming growth factor beta 1</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRAP6</td>
<td>thrombin receptor activating peptide 6</td>
</tr>
<tr>
<td>TSG6</td>
<td>tumor necrosis factor-stimulated gene-6</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
</tbody>
</table>
CHAPTER I

BACKGROUND

1.1. The Extracellular Matrix

Although cells represent the major component of living tissues, a significant volume of the tissues is occupied by an extracellular space known as the extracellular matrix (ECM). A wide variety of molecules constitute the ECM, including proteins and polysaccharides. Most of these molecules are organized at the surface of the cells that produce them forming a network between cells. The diversity of molecules that form the ECM varies depending on tissue type. Whereas the matrix in bones is calcified to produce hard structures, the matrix of the cornea is soft and transparent [1,2].

Aside from its classical role in structure and support, ECM also has a major role in cell regulation, including cell function, migration, proliferation and survival. The ECM is composed mainly of two types of macromolecules. The first type is the fibrous proteins that have roles in both structure and adhesion. These include elastin, which provides elasticity; collagen, which forms fibers that provide strength and organization to the
matrix; and fibronectin, which assists in cell attachment [1]. The second is a class of large polysaccharides, known as glycosaminoglycans (GAGs). The fibrous proteins are typically entrenched in a gel-like structure formed by the GAGs. The organization allows for the blood and cells to interact readily, aiding in the fast diffusion of molecules. Most GAGs exist in proteoglycans, a class of carbohydrates covalently linked to protein cores. The only exception is hyaluronan (HA), which does not include a protein as a core component [3,4].

1.1.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) represent a major constituent of the ECM, although they can also be found intracellularly. The name refers to the presence of amino monosaccharides and other monosaccharides in a polymeric structure. With the exception of HA, GAGs can be sulfated and covalently linked to a protein. This group of macromolecules is made up of HA, dermatan sulfate (DS), keratan sulfate (KS), chondroitin, chondroitin sulfate (CS), heparin, and heparan sulfate (HS). These proteoglycans are composed of a core protein and chains of linear GAGs covalently linked to the protein. GAGs are made up of chains of disaccharides; each disaccharide is composed of an amino monosaccharide (N-acetylglucosamine, or N-acetylgalactosamine) and a non amino monosaccharide (a uronic acid, or galactose). Whereas some proteoglycans have only one linked GAG chain, others can have many GAG chains attached [5,6].

GAGs in the ECM demonstrate a wide range of functions. For example, CS and DS proteoglycans are extremely hydrophilic, which aides in forming hydrated matrices
that occupy the space between cells. The large volume HA occupies in solution creates a structure that absorbs compressive loads in cartilage [7]. Basement membrane organization is another major function of GAGs [8]; they create a suitable environment for epithelial cells to proliferate and differentiate. KS, on the other hand, plays a crucial role in the cornea. The special arrangement of KS and collagen in the cornea permits the light to pass without dispersion [9].

Not only do GAGs play a role in structure and support, but they also have important regulatory roles. GAGs can bind cytokines and chemokines; they interact with growth factors, thus affecting signaling pathways; and they act as receptors for endocytosis. In addition, GAGs, in cooperation with adhesion receptors, facilitate the interaction between cells as well as cell motility and migration. The interactions between GAGs and various protein ligands lead to numerous physiological outcomes. A good example is the role heparin plays in anticoagulation. Injected heparin is widely used as an anticoagulant due to its binding capacity to antithrombin, resulting in its activation, and to P-selectin, resulting in its blockade, which consequently results in anticoagulation [10].

1.2. Hyaluronan

1.2.1. Hyaluronan Structure

Hyaluronan (HA) is a ubiquitous, non-sulfated, unbranched GAG and the largest polysaccharide produced in vertebrates. It is the only GAG that does not have a core protein component. HA chain is made up of repeating disaccharides; each disaccharide is composed of D-N-acetylglucosamine and D-glucuronic acid linked by alternating β-(1,4) and β-(1,3) glycosidic bonds (figure 1.1). HA can become very large reaching up to $10^7$
Dalton. HA is also highly polyanionic due to the carboxyl groups of the glucuronic acid residues, which gives it a random coil structure in solution [11]. One of the most important characteristics of HA is its high hydrophilicity, which can result in a very viscous environment when water is bound to HA. This property is clearly found in the vitreous humor of the eye and in joints, where HA is abundant. For example, HA, in synovial fluid of joints, aids in load distribution during motion, in lubrication, and in protection of the surfaces of cartilage [12].
Figure 1.1: The disaccharide that represents the building block of hyaluronan.

Hyaluronan is made up of repeating disaccharides of D-N-acetylglucosamine and D-glucuronic acid linked by alternating β-(1,4) and β-(1,3) glycosidic bonds. N = 2 – 20,000.
1.2.2. Hyaluronan synthesis

Three mammalian HA synthase enzymes (HAS) have been identified: HAS1, HAS2, and HAS3; their structure is well-conserved among various mammalian species. Structurally, they contain seven membrane spanning regions: two at the N-terminus and five at the C-terminus. Whereas HAS3 synthesizes HA with a size range of $10^5$-$10^6$ Dalton, HAS1 and HAS2 are capable of producing much larger HA polymers. HA is synthesized, uniquely and contrary to the other GAGs that are synthesized in the Golgi apparatus, at the inner surface of the cell membrane by one of the HAS enzymes, where UDP-$N$-acetylglucosamine and UDP-glucuronic acid are added alternately to the reducing end of the HA chain being synthesized. The growing HA molecule then gets translocated extracellularly through the membrane by HAS. Studies have shown that HAS2 is responsible for the majority of HA synthesis compared to HAS1 and HAS3 and that knocking down HAS2 in mice is embryonically lethal due to severe cardiovascular defects. On the other hand, HAS1/3 knockout mice develop normally. The activity of HAS enzymes can be regulated at the transcriptional level or at the posttranslational level by phosphorylation, O-GlcNAcylation, or ubiquitination. HAS activity can also be regulated by the cellular content of the UDP- $N$-acetylglucosamine and UDP-glucuronic acid. Another mechanism by which HAS2 is regulated is dimerization. HAS2 can form homodimers or heterodimers with HAS3 in the plasma membrane, which can have an effect on its activity [13-15].

1.2.3. HA binding proteins
Under many conditions, HA exists in the body bound to one of its protein partners. Most of HA binding proteins contain a motif known as the link module, which is a protein fold that binds specifically to HA. The fold is composed of two α-helices and two triple-stranded antiparallel β sheets. Both the hydrogen bond, formed between HA and the aromatic amino acids of the link module, and the ionic interaction between the carboxylic acid groups of the glucuronic acid residues of HA and the positively charged amino acid residues of the link module facilitate the binding of HA to its binding proteins [16]. HA binding proteins that contain a link module can be either associated with cell surface, such as CD44, lymphatic vessel endothelial hyaluronan receptor (LYVE-1), and hepatic hyaluronan clearance receptor (HARE), or secreted molecules, such as versican and aggrecan. Some HA binding proteins do not have the link module; this list includes receptor for HA-mediated motility (RHAMM) and inter-α-trypsin inhibitor (IαI).

CD44 is a ubiquitously expressed glycoprotein by most mammalian cells and is considered the major cell surface receptor for HA. At least 8 isoforms of CD44 have been identified, which are encoded by alternate splicing of the CD44 gene. The cytoplasmic end of CD44 has a signaling domain, whereas its extracellular domain has the link module. It has been shown that the binding capacity of CD44 to high molecular weight HA is higher than its capacity to bind low molecular weight HA. Importantly, CD44 can respond to HA binding through the interaction of its cytoplasmic tail with many regulatory proteins, including kinases and cytoskeletal components [17].

1.2.4. Hyaluronan catabolism
The catabolism of HA in humans occurs by endo-β-N-acetylhexosaminidase enzymes known as hyaluronidases (HYALs). The six identified hyaluronidases in mammals are clustered on two chromosomes: HYAL1, HYAL2, and HYAL3 are clustered on chromosome 3 at 3p21.3, whereas HYAL4, HYALP1, and SPAM-1 are located likewise on chromosome 7 at 7q31.3. Out of the six HYAL enzymes, HYAL1 and HYAL2 are the only somatically active HA degrading enzymes in humans. HYAL3, although widely expressed, does not appear to have HA degrading activity. SPAM1 (Sperm Adhesion Molecule), on the other hand, is found only in the sperm and is necessary for fertilization; it cleaves HA from the cumulus mass surrounding the ovum, which facilitates the penetration of the sperm. HYAL4 shows only chondroitinase activity and HYALP1 is a pseudogene that is not translated in humans [18,19].

1.2.4.1. HYAL1

HYAL1 was identified as an acid-active enzyme in serum in 1967 by De Salegui et al. [20]. However, it was not cloned and sequenced until 1997 by Frost et al. [21]. HYAL1 is a 57-kDa protein that is 40% homologous to SPAM1 AND 73% homologous to its mouse ortholog. Its catalytic activity results in HA tetrasaccharides as products. Interestingly, a genetic deficiency of HYAL1 has been reported in humans and named Mucopolysaccharidosis IX. Although patients have a normal phenotype, there is some growth retardation, bilateral peri- and intra-articular soft tissue masses and generalized cutaneous swelling. Importantly, their level of circulating HA is extremely high and their histiocytes are filled with many large membrane-bound vacuoles, suggesting that the disease is a lysosomal storage disease and that HYAL1 is a lysosomal enzyme [22].
1.2.4.2. HYAL2

In 1998, Lepperdinger et al. identified HYAL2 as a lysosomal enzyme after expressing green fluorescent protein-tagged HYAL2 in a rat glioma cell line. They also showed that whereas HYAL2 protein and mRNA were detected in most human tissues, including heart, liver, kidney, and placenta, they were absent from brain tissues. Additionally, these studies showed that HYAL2 is an acid-active enzyme with optimum activity below pH of 4 and that it is capable of degrading high molecular weight HA into about 20-kDa HA polymers. Interestingly, HYAL2 did not further degrade the 20-kDa products nor did it degrade 20-kDa HA fragments isolated from vitreous humor [23].

Later studies on HYAL2 have shown and confirmed, conversely, that HYAL2 is in fact a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein. A GPI-anchored protein is typically attached to the GPI by its carboxyl terminus and is located in the outer leaflet of the lipid bilayer facing the extracellular surroundings. Although recombinant HYAL2 shows weak activity compared to HYAL1 and SPAM1, significant HAase activity can be detected in cells overexpressing the GPI-anchored HYAL2 [24]. Additionally, a soluble form of HYAL2, prepared by inserting a stop codon into HYAL2 cDNA at the position of the GPI-anchor cleavage site, demonstrated similar activity at low pH. This activity was significantly decreased by mutating the amino acids corresponding to the active site residues known in the bee venom and SPAM1 hyaluronidases, suggesting that the HAase activity site of HYAL2 is similar to the activity sites in other hyaluronidases. However, the soluble form of HYAL2 digested the 20-kDa HA, in contrast to the previously reported GPI-anchored HYAL2, which was incapable of such activity [25].
1.2.4.3. HYAL2 as a virus receptor

Different non-HA-degrading functions of HYAL2 have been reported. Notably, HYAL2 plays a role as a virus receptor. HYAL2 has been shown to act as a cell-entry receptor for Jaagsiekte sheep retrovirus (JSRV), a virus that causes lung cancer in sheep and goats, and enzootic nasal tumor virus (ENTV), a virus that causes nasal tumors in these animals. The envelope (ENV) proteins of the two retroviruses play two major functions: they contribute to tumorogenesis by transforming fibroblasts and epithelial cells, and they facilitate the entry of the virus into cells through binding specifically to HYAL2, which suggests that HYAL2 may be playing a major role in cell transformation and oncogenesis. Importantly, HYAL2 is localized to a lung cancer tumor suppressor locus on chromosome 3p21.3. Binding of ENV to HYAL2 likely damages the tumor suppression activity of HYAL2, resulting in tumor formation [24-26].

1.2.4.4. A model for HA degradation

The turnover of HA occurs in a very rapid rate in the body. HA is present normally in high amounts in different bodily tissues and fluids, including the joint, the eye vitreous, the umbilical cord, and amniotic fluid. High levels of HA are also present in proliferating tissues and tissues undergoing repair. As a result, the rapid catabolism of HA, through the activity of HYAL enzymes, represents a major mechanism by which HA levels are regulated in the body [27]. Studies have shown that HA degradation is dependent upon the classical HA binding receptor CD44 and involves mainly HYAL1 and HYAL2.
Bourguignon et al. have shown that the degradation of HA by HYAL2 is CD44-dependent. In human breast tumor cell line, they investigated the role of CD44 in HA degradation. They showed that the binding of HA to CD44 activates Rho kinase, which, in turn, causes the phosphorylation, and activation, of Na\(^+\)-H\(^+\) exchanger (NHE1). The activity of NHE1 promotes intracellular acidification and creates an acidic extracellular matrix environment. The created acidic environment provides optimum condition for HYAL2 to degrade HA at the cell surface [28].

Other studies have also confirmed the role of CD44 in HA degradation. Harada et al. thoroughly investigated the roles of HYAL1, HYAL2, HYAL3, and CD44 in the degradation of HA in a human embryonic kidney cell line. They found that HYAL1 cannot degrade HA in cells lacking CD44 and that its activity in cells expressing CD44 is primarily intracellular. Conversely, the activity of HYAL2 was strictly extracellular but also dependent upon CD44, which exists on the cell surface along with HYAL2. HYAL3, on the other hand, demonstrated no hyaluronidase activity [29].

In the light of these reports as well as other studies of HA catabolism, the accepted model for HA degradation in tissues of vertebrates involves four enzymes: HYAL1, HYAL2, and two β-exoglycosidases (figure 1.2). Degradation of HA starts at the cell surface. CD44 binds HA extracellularly and mediates the process by creating an acidic microenvironment. Then, the acid-active enzyme HYAL2 degrades large HA polymers into 20-kDa polymers. These HA products get internalized into the lysosomes, where they get degraded by HYAL1 into tetrasaccharides before N-acetyl glucosaminidase and glucuronidase enzymes further degrade them into monosaccharides.
Figure 1.2. Representation of a proposed model for HA degradation in cells.

Degradation of HA starts at the cell surface. CD44 binds HA extracellularly and mediates the process by creating an acidic microenvironment. Then, the acid-active enzyme HYAL2 degrades large HA polymers into 20-kDa polymers. These HA products get internalized into the lysosomes, where they get degraded by HYAL1 into tetrasaccharides before N-acetyl glucosaminidase and glucuronidase enzymes further degrade them into monosaccharides [18].
1.2.4.5. KIAA1199

Recently, in 2013, a new contributor to HA degradation was discovered. Yoshida et al. have reported that KIAA1199, which is a gene related to deafness but with unknown function, has a principle role in the binding and the depolymerization of HA. The procedure by which the researchers have identified KIAA1199 as a HA degradation-related protein was remarkable. They first treated cultured human skin fibroblasts with various stimulators and monitored HA degradation. They found that histamine treatment resulted in increased HA degradation whereas transforming growth factor beta 1 (TGF-β1) treatment resulted in decreased HA degradation. Then, using microarray analysis, they identified 25 genes that were both up-regulated by histamine and down-regulated by TGF-β1. After knocking down all of the 25 genes in the cultured cells and monitoring HA degradation following each knockdown, they found that knocking down KIAA1199 resulted in abolished HA degradation [30].

Additionally, when KIAA1199 was expressed in cells lacking HA-cleaving activity, the transfected cells acquired the ability to cleave HA. Importantly, out of all GAGs tested for their binding ability to KIAA1199, only HA demonstrated HA-binding activity. The specific binding of KIAA1199 to HA contributed to the catabolism of HA via the clathrin-coated pit pathway. To assess the possibility that KIAA1199 plays a role in arthritic diseases, which are characterized by reduced concentration of large molecular weight HA and accumulation of low molecular weight HA, the researchers compared the HA-degrading activity of arthritic synovial fibroblasts to healthy synovial fibroblasts and found that arthritic fibroblasts demonstrated higher activity than the healthy ones and that this activity was abolished by knocking down KIAA1199 in these cells. Importantly,
KIAA1199 gene expression was significantly higher in synovial tissues of arthritis patients compared to non-arthritic controls [30]. In addition, the group has shown that the mouse ortholog of KIAA1199 also possess HA binding and degrading activity [31]. Many studies have reported that KIAA1199 is also found to be overexpressed in different cancers including breast and colorectal cancers [32].

1.2.4.6. Non-enzymatic means of HA degradation

In addition to the enzymatic processes that cleave HA, HA can be degraded by oxidation reactions; particularly, by reactive oxygen species (ROS) and free radicals. The damaging outcome of ROS is observed in many diseases. For example, ROS, resulting from the inflammation of the joints in patients with arthritic diseases, can cause damage to the synovial fluid. Specifically, ROS can destroy synovial fluid HA, which is a major component of the synovial fluid and contributes to the lubrication and the smooth movement of the joint. A reduction in the molecular weight of synovial fluid HA in patients with arthritis has been reported in many studies. Ultimately, degraded synovial fluid results in stiff and painful joints [33].

HA degradation by ROS may also explain the oxidative stress-induced submucosal gland cell hypertrophy and hyperplasia observed in chronic bronchitis. Casalino-Matsuda et al. have shown that ROS-induced HA depolymerization results in the activation of tissue kallikrein, a protease bound to HA in airway submucosa, the release of mature epidermal growth factor, and, consequently, the activation of epidermal growth factor receptor (EGFR). The activation of EGFR then contributes to the development of gland hyperplasia in the airways. Studies from the same group have
shown that ROS-induced HA depolymerization results, through a CD44 and EGFR-dependent pathway, in increased MUC5B, one of the major mucins present in the secretions of airways from patients with asthma cystic fibrosis, and chronic obstructive pulmonary disease [34].

Not only can ROS induce HA fragmentation directly, but it also can contribute to HA degradation indirectly. For example, Monzon et al. have shown that ROS can cause an increase in the expression and activity of HYAL2 through a p38 MAPK-dependent signaling pathway in normal human bronchial epithelial cells. The group has also shown, importantly, that in the tracheal tissues and secretions in smokers, who represent a model for chronic oxidative stress exposure, HYAL2 is highly expressed, along with an increased concentration of low molecular mass HA [35].

1.2.5. HA function is size-dependent

Accumulating evidence from studies published in the last 20 years has confirmed that the molecular weight of HA is critical in determining the biochemical and cellular roles of HA. An increasing body of literature suggests that different sizes of HA exert a wide spectrum of functions [36,37]. In tissues under normal conditions, HA is present in its high molecular weight (HMWHA) form with an average size range of 1-5 X 10^6 Dalton. HMWHA functions as a structural, hydrating polymer due to its hydrophilic properties. Beyond its role as a space filling, lubricating molecule, HMWHA is also known to be anti-inflammatory and anti-angiogenic. Also, HMWHA is shown to mediate cancer resistance and to prevent cytolysis. Conversely, low molecular weight HA has been shown to have pro-inflammatory and pro-angiogenic effects.
In their widely publicized report published in *Nature*, Tian et al. showed that extremely large molecular weight HA mediates cancer resistance in naked mole rats. Naked mole rats are exceptional rodents as they have a lifespan exceeding 30 years. This longevity is thought to be attributed to the fact that naked mole rats are resistant to developing cancer. The group reported that fibroblasts from naked mole rats synthesize and release a very large molecular weight HA (up to $12 \times 10^6$ Dalton), which is more than five times larger than HA detected in human and mouse fibroblasts. Additionally, they found that the unusually large HA that is accumulating in the animal tissues is due to a unique form of the enzyme HAS2 (two asparagine amino acid residues that are 100% conserved in mammals were replaced with two serines in the naked mole rat HAS2) and to the decreased activity of the enzyme HYAL2. Importantly, knocking down HAS2, or overexpressing HYAL2 in these animals made them susceptible to developing tumors [38].

A size-dependent function of HA in angiogenesis has also been reported [39]. It has been shown that HMWHA at physiologic concentrations has anti-angiogenic properties. Intact HA binds to CD44 on the cell surface, which can block crucial intracellular signaling pathways that result in cell proliferation. This effect can prevent endothelial cells from proliferating and thus impact angiogenesis. On the other hand, LMWHA fragments were shown to have pro-angiogenic effects by stimulating endothelial cell proliferation. LMWHA can compete with native HMWHA in binding to endothelial cell CD44. These small fragments can mediate intracellular signaling pathways that facilitate cell proliferation. LMWHA can also stimulate the expression of matrix metalloproteinase (MMP) 2 and 9, which assists in cell invasion through ECM.
barriers to and mediates vessel development and growth. These MMPs can also induce angiogenesis by activating TGF-β [39]. HA fragments also interact with RHAMM, which results in the modification of endothelial cell cytoskeleton, aiding in their migration. LMWHA/RHAMM interactions also affect wound healing. Savani et al. have shown that endothelial cell motility is dictated by binding of HA to RHAMM [40].

1.2.6. HA in inflammation

Elevated levels of accumulated HA have been observed in many inflammatory diseases. For example, high levels of HA in joint tissues of patients with rheumatoid arthritis have been reported. Additionally, many studies have reported on the increased HA deposition in inflammatory diseases of the liver. Whereas the concentration of HA in the healthy liver is low, its concentration significantly increases in fibrotic liver, leading to increased levels of serum HA. As a result, the level of circulating HA has been proposed as a biomarker for cirrhotic liver disease, for monitoring liver function, for assessing liver fibrosis, and for diagnosing chronic viral hepatitis C [41]. HA levels also increase in patients with inflammatory diseases of the lung, including asthma [42]. Studies have also shown that an upregulation of HA occurs in the intestines of patients with Inflammatory Bowel Disease (IBD) [43].

1.2.6.1 Role of HMWHA in inflammation

Many reports have shown that HMWHA has a crucial role in regulating inflammation and that it has an anti-inflammatory effect, in addition to its function as a structural, hydrating polymer. Large HA polymers have a role as molecules that indicate the integrity of tissues; they also control the cellular inflammatory responses [44].
HMWHA can protect from T-cell-mediated liver injury and bleomycin-mediated lung injury in mice and it can promote the suppressive effects of regulatory CD4+ CD25+ T cells [45-47].

Nakamura et al. have shown that HMWHA, and not LMWHA, can prevent T-cell-mediated liver injury by decreasing proinflammatory cytokines. The group injected mice with different sizes of HA ranging from 250 to 1200 kDa before inducing liver injury by administrating the mice with concanavalin A, which induces a T-cell mediated liver injury and affects the production of cytokines. They found that pretreatment with HMWHA, specifically, decreased the levels of alanine aminotransferase in plasma that were elevated due to injury. Additionally, they found that HMWHA treatment also decreased cytokine levels, including tumor necrosis factor-alpha (TNF-α) and interleukin 4, and chemokine levels, including macrophage inflammatory protein 2. Collectively, the researchers concluded that HMWHA plays a protective effect and can prevent T-cell-mediated liver injury [45].

In their 2002 report in the journal *Science*, Teder et al. showed that HMWHA and its binding partner CD44 have a protective role against lung inflammation. The group has administered wild type and CD44-deficient mice intratracheally with bleomycin, which is used to induce a lung injury that is characterized by inflammation of alveolar interstitium and the accumulation of ECM. The group found that CD44 and HMWHA contribute to the resolution of inflammation [46]. Additionally, Bollyky et al. have shown that HMWHA, not LMWHA, has the ability to induce the suppressive effects of regulatory T-cells (r-Tc). The group proposed that the state of HA in the ECM provides a signal to r-Tc and T-cells (Tc) and provides a link between the innate and adaptive
immunities. Whereas both HMWHA and LMWHA were able to bind r-Tc, only HMWHA was able to induce the suppressive effect on these cells, which is to suppress the proliferation of Tc. Interestingly, the group has shown that HMWHA can by itself suppress the proliferation of Tc, directly [47].

1.2.6.2 Role of LMWHA in inflammation

Molecules that can mediate and perpetuate an immune response in the absence of an infectious agent are known as Damage Associated Molecular Patterns, or DAMPs, as opposed to PAMPs (Pathogen Associated Molecular Patterns), which initiate a pathogen-related inflammatory response. Fragmentation of intact HMWHA through the activity of the HYAL enzymes, particularly HYAL2, or by ROS species, results in the formation of LMWHA, or HA fragments, which function as DAMPs. Many reports have shown that fragmented HA is a pro-inflammatory molecule and is capable of signaling cellular responses through specific receptors, including CD44 and toll-like receptors (TLR) 4 and 8 [37,44].

Many reports have shown that HA fragments can activate immunocompetent cells, including dendritic cells and macrophages. Termeer et al. have shown that HA fragments induce dendritic cell maturation through a TLR4-dependent mechanism. They showed that dendritic cells expressing a mutant form of TLR4 were not activated by HA fragments, whereas dendritic cells lacking TLR2 were. Additionally, blocking TLR4 abolished the production of HA-fragment-induced TNF-α. The HA/TLR4-dependent dendritic cell maturation required the activity of p38-MAPK and P42/44-MAPK and
nuclear translocation of NF-κB, consistent with the signaling cascade involving TLR4 [48].

In monocytes, HA fragments were able to stimulate the production of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP1) through both TLR4- and CD44-dependent mechanisms. Yamawaki et al. have shown that peripheral blood mononuclear cells (PBMCs) specifically expressed TLR4 and CD44, among other HA receptors. LMWHA, but not HMWHA, stimulated the production of IL-6 and MCP-1 production in PBMCs. When cells were incubated with antibody against CD44, the production of IL-6 and MCP-1 in LMWHA-stimulated PBMC was significantly reduced. Additionally, the production of IL-6 by cells lacking TLR4 and CD44 was reduced [49].

Recent studies have shown that HA fragments can also induce innate host defense responses at the intestinal epithelium. Hill et al. have shown that HA fragments with a specific size of 35-kDa (HA-35), but not larger, can promote the expression of an innate antimicrobial peptide known as human β-defensin 2 (HβD2) in intestinal epithelial cells. The group treated colonic epithelial cells with different HA sizes and found that only the 35-kDa HA preparation induced the expression of HβD2 in these cells. Additionally, the group tested the expression of HβD2 in vivo in response to HA-35 treatment. They found that oral administration of HA-35 induced the expression of the mouse ortholog of HβD2 in the colonic epithelium of both wild-type and CD44-deficient mice but not in TLR4-deficient mice, suggesting that the induction of HA-35-mediated innate host defense depends upon TLR4, but not CD44 [50].

1.2.6.3. Pathological HA cables
A special type of pathological HA that also exists in the body, mostly under inflammatory conditions, is referred to as HA cables. HA cables are large HA structures decorated with proteins, including the heavy chains of IαI, tumor TNFα-stimulated gene 6 (TSG6) and versican. The major feature that functionally distinguishes HA cables from other non-pathological HA structures is their ability to bind leukocytes. These leukocyte adhesive HA matrices have been reported in many inflammatory diseases, including intestinal tissues of IBD patients, lung tissues of asthmatic patients, lung tissues from idiopathic pulmonary hypertension patients, and synovial fluid of patients with arthritic disease [51,52]. Additionally, HA-cable formation was accomplished in vitro by inducing endoplasmic reticulum stress or by virus infection in intestinal and airway smooth muscle cells [53,54]. Figure 1.3 shows intestinal mucosal smooth muscle cells before (1.3A) and after (1.3B) they were stimulated with polyI:C, which is a double stranded RNA that mimics a virus infection. Whereas most HA in non-stimulated cells appears to be intracellular, HA in polyI:C-stimulated cells appears to be forming cable-like structures that span the surfaces of multiple cells.

HA cables have been shown to be associated with heavy chains of IαI [51]. IαI is a plasma proteoglycan that is produced in the liver. It consists of a single chondroitin sulfate chain and three proteins covalently linked to the chondroitin sulfate chain: heavy chain 1, heavy chain 2, and bikunin, which exerts the trypsin inhibition function of IαI [55]. During inflammation, IαI can leak from serum into the extravascular spaces as a result of increased vascular permeability. The exposure of IαI to the extracellular matrix allows it to function as a heavy chain donor to HA. TSG-6, an enzyme and a HA binding protein, facilitates the transfer of heavy chains 1 and 2 from IαI to HA to form the
leukocyte-adhesive HA structure. Importantly, TSG-6 expression increases in the inflamed tissues, which emphasizes the role HA cables play in inflammation [56].

Figure 1.3: HA cables on the surface of poly:I:C-stimulated mucosal intestinal smooth muscle cells (M-SMCs). (A) M-SMCs before they were stimulated with polyI:C, which is a double stranded RNA that mimics a virus infection. Most HA (green) appears to be intracellular. (B) M-SMCs after they were stimulated with polyI:C. HA (green) appears to be forming cable-like structures that span the surfaces of multiple cells. green = HA, blue = M-SMC nuclei.
1.2.6.4. HA cables in intestinal inflammation

In 1999, de la Motte et al. showed for the first time that mononuclear leukocytes bind specifically to HA on the surface of colonic mucosal smooth muscle cells (M-SMCs) that were infected with a virus. The group wanted to test the response of cultured M-SMCs to virus infection because IBD correlates with virus infections-related flares and with increased intestinal mucosal leukocytes. They found that adhesion of leukocytes to M-SMCs infected with syncytial virus, measles virus, or polyI:C increased significantly compared to non-infected cells. Interestingly, not only did stimulated M-SMCs produce more cell-surface HA, but also treatment of these cells with hyaluronidase significantly reduced leukocyte adhesion. Additionally, blocking CD44 on leukocyte surfaces decreased adhesion to polyI:C-treated M-SMCs. Collectively, the data showed that mononuclear leukocytes bind specifically to HA of inflamed cells via CD44 [53].

In a follow-up study [51], the same group showed that treating M-SMCs with polyI:C caused HA to form coat-like structures within 3 hours of treatment, which turned into long cable structures spanning multiple cells within 17 hours of treatment. Importantly, these cable structures were shown to be the principle mediator of leukocyte adhesion. Additionally, they showed that IαI is a major component of both the coat-like and cable structures and that culturing cells in the presence of an antibody against IαI prevented cable formation and, consequently, leukocyte adhesion. The group also showed for the first time that inflamed intestinal tissues from patients with IBD have elevated HA expression, and that the elevated HA is associated with IαI [51].
It was still unclear whether the accumulation of HA precedes and promotes intestinal inflammation, or it is the result of inflammation. To answer this critical question, Kessler et al. used a mouse colitis model and investigated changes in the deposition of HA in the intestine and how these changes associated with the changing pathological architecture of intestinal tissues over time (figure 1.4). Within 3-7 days of oral administration of dextran sulfate sodium (DSS), a heparin-like polysaccharide that induces intestinal inflammation, colon HA deposition changed significantly in the intestines of the mice. In fact, HA deposition into colonic blood vessels was detected before any detected inflammatory infiltrate. Importantly, the group reported that HA deposition occurs also in the blood vessels of IBD patients’ colons and that, in vitro, human intestinal endothelial cells can form HA cables that are leukocyte adhesive in response to TNF-α, a proinflammatory cytokine. Their data provided evidence that the deposition of HA is an early event in inflamed intestinal tissue and that it precedes and maybe promotes the infiltration of leukocytes [43].
Figure 1.4: Detection of HA structures within the blood vessels of an inflamed colon of a mouse and a human IBD patient. (A) Mucosal blood vessels in a section of distal colon from a mouse treated with DSS have HA (green) within the vessel lumen. (B) Colon sections from a Crohn’s disease patient show abundant HA staining (green) throughout the mucosa and submucosa. (C) A blood vessel reveals HA structures within the intestinal blood vessels, in proximity to leukocytes (red). Nuclei are blue [43].
1.3. Inflammatory Bowel Disease

Inflammatory Bowel disease (IBD) is a group of chronic inflammatory conditions of the intestines. Over 1.5 million people are affected with IBD in the United States and the incidence of the disease is increasing around the world. Although IBD can occur at any age, the peak onset for the disease occurs in people 15-30-year old. People who suffer from IBD put up with a lifelong of medical attention and persistent symptoms, including vomiting, diarrhea and rectal bleeding. While the etiology of IBD is not completely known, genetic, immunologic, and environmental factors appear to contribute to the disease. IBD is also widely associated with a westernized lifestyle [57].

IBD consists of two main types: Crohn’s disease and ulcerative colitis, among other less common inflammatory diseases. Whereas ulcerative colitis is characterized by lesions in the large intestine and the rectum as well as inflammatory lesions restricted to the bowel mucosa, Crohn’s disease can affect any part of the gastrointestinal tract from the mouth to the anus and the inflammation may extend to the bowel wall. In IBD, chronic inflammation of the intestine leads to damaged epithelium, which results in a loss of its function; instead, a fibrotic non-functional tissue forms in place of the inflamed mucosa, which leads to abdominal pain, malnutrition, and poorly controlled bowel. In many cases, IBD patients require surgery when they end up with a malfunctioning intestine. None of the currently applied treatments for IBD cure the primary pathological processes that lead to the disease; instead, they reduce symptoms [58].

1.4. Platelets
Platelets are small (about 2 µm in diameter), anucleate, circulating cells that are derived from megakaryocytic precursors in the bone marrow. They were first described in 1862 as novel blood elements that have roles in haemorrhage and thrombosis. The principle physiological functions of platelets are to recognize any damage or injury that occurs in the endothelium of blood vessels, act to “seal off” the damage, and prevent bleeding by accumulating at the site of the vessel injury and initiating the clotting of the blood. Upon vascular damage, platelets get activated after they bind to the collagen or to other exposed ECM proteins, which promotes the release of molecules that signal the recruitment, and activation, of more platelets [59].

The precursors of platelets, the megakaryocytes, are large cells that reside in the bone marrow. Each megakaryocyte can release thousands of platelets. First, a megakaryocyte undergoes a dramatic rearrangement of its cytoplasm to produce elongated and highly branched structures known as proplatelets. Proplatelets extend into the blood vessels where they undergo further fragmentation into platelets [60]. Circulating, resting platelets have a discoid oval shape and typically a smooth surface. However, upon activation, platelets change shape into spheres with spiny looking pseudopods (figure 1.5). Importantly, activated platelets release numerous adhesive and clotting proteins from their granular stores to the surrounding environment. These molecules can be soluble, associated with the platelet surface, or packaged in microparticles [61]. For example, P-selectin is an adhesion molecule that is stored inside \( \alpha \)-granules of resting platelets. However, the protein translocates to platelet surface upon activation to exert its adhesion function.
Platelets have three main storage compartments: α-granules, dense granules and lysosomes, in addition to their mitochondria and dense tubular system. The most abundant storage compartments are α-granules. Their protein contents include coagulation factors, adhesive proteins, and inflammatory and angiogenic mediators. Interestingly, studies have shown that functional coclustering of proteins within different α-granules is possible. For example, Italiano et al. have identified two classes of α-granule: one that contains anti-angiogenic factors and another that contains pro-angiogenic factors [62]. Dense granules, on the other hand, are present in a fewer number in platelets and are the principle storage compartments of small molecules such as serotonin, ADP, and calcium, which is necessary in the coagulation cascade [63].
Figure 1.5: Transmission electron microscopy images of resting and activated platelets. Non-activated platelets have a discoid shape with smooth surface, whereas activated platelets have more spherical shape and arm-like structures, or pseudopods. The figure also shows the granules inside platelets.
1.4.1. Platelet role in inflammation

Beyond their major role as cellular mediators of thrombosis and hemostasis, accumulating evidence suggest that platelets play an important role as immune cells that can initiate many inflammatory processes. With more than a trillion platelets circulating the human body, platelets represent the most numerous cell type with an immune function. Platelets have been linked to the pathogenesis of several inflammatory diseases, including rheumatoid arthritis, atherosclerosis, malaria infection, and transplant rejection. Depending on the situation, platelets can either have a protective immune effect or a pro-inflammatory effect [64].

Some of the components of platelet α-granules (e.g. platelet factor 4) have no known function in thrombosis, but rather they are known to act as chemokines and cytokines with the ability of recruiting immune cells and driving inflammation [65]. Components of dense granules can also have immune functions, in addition to their thrombotic functions. For example, platelets release their dense granular content of ADP and ATP. ADP is known to activate P2Y$_{12}$ on dendritic cells, resulting in increased antigen endocytosis and processing, whereas ATP can signal through T-cell P2X7 resulting in the differentiation of CD4$^+$ T helper cell into a proinflammatory T-helper 17 cell [66,67].

Studies have also shown that platelets can induce acute phase response (APR), which is the earliest response to infection during which the growth of microbes is inhibited by specialized proteins and the infection is limited by trapping pathogens within local blood clots. Platelets can serve as a major source of interleukin-1β (IL-1β), which
is cytokine that is known to induce APR. Interestingly, IL-1β is not found in resting platelets; instead, platelets have IL-1β pre-mRNA and the required machinery to process it into mRNA and synthesize and release a functional IL-1β protein upon activation [68]. Additionally, it has been shown that platelets get activated postinfection in a mouse model of malaria and that platelet IL-1β has a role in inducing APR in these animals [69].

Platelets store numerous inflammatory mediators, which can be released upon activation. For example, platelet-expressed CD154 can promote leukocyte recruitment to sites of inflammation through interaction with endothelial cell-derived CD40. Platelets also express a number of the toll-like receptor (TLR) family members, such as TLR4 and TLR9. Platelet TLR4, for example, binds bacterial LPS and can present bacterial products to neutrophils; whereas TLR9 expressed on activated platelets can mediate bacterial DNA sequestration [70].

1.4.2. Platelets in IBD

Abnormalities in platelets have been reported in both types of IBD: Crohn’s disease and ulcerative colitis. Perhaps the most notable platelet abnormalities in IBD are that platelets circulate the periphery in a highly activated state and the presence of platelet aggregates within mucosal microthrombi in the mucosa of the intestines of patients with IBD [71,72]. The role platelets may have in the pathogenesis of IBD is unclear. However, there is accumulating evidence that platelets have a role as potent pro-inflammatory cells. Activated platelets release biological mediators that induce inflammation through processes similar to those used by immune cells, which have a known role in the
pathogenesis of IBD. Therefore, understanding the roles platelets play in IBD might be crucial to the understanding of the pathogenesis of this enigmatic disease.

It is established that patients with an active state of IBD have reactive thrombocytosis (higher than normal platelet count) and that their platelet count correlates well with the severity of the disease. However, the reason behind the increased number of circulating platelets in IBD patients is still unclear [73]. Additionally, reports have shown that platelets of IBD patients are smaller in size, with mean platelet volume inversely proportional to the levels of C-reactive protein, which is an inflammatory marker. Other reports have shown also that platelets of IBD patients have increased density due to their increased granular content [74].

Several studies have reported on the correlation between platelet activation and IBD. For example, it has been reported that IBD patients’ platelets undergo spontaneous aggregation in vitro in comparison to control platelets. Additionally, platelet aggregates are detected in vivo in patients with IBD. Other reports have shown that IBD platelets are more sensitive to in vitro activation by an agonist compared to controls. Importantly, the activation state of platelets of IBD patients in peripheral circulation is elevated, indicated by the increased expression of platelet activation markers such as P-selectin. Moreover, increased levels of some platelet activation-related proteins (e.g. platelet factor 4) have been reported in the circulation of IBD patients, suggesting the increased activation state of platelets in IBD patients [75,76].

1.4.3. Platelet HYAL2
Many reports have shown that the degradation of HA can result in the generation of small sized HA fragments capable of inducing angiogenic and inflammatory responses. In 2009, de la Motte et al. reported that platelets and their megakaryocytic precursors contain mRNA and protein of HYAL2, one of the two somatically active hyaluronidases commonly found in the human body, without evidence of HYAL1. Additionally, they showed that platelets can cleave HA cables from the surfaces of activated human intestinal mucosal endothelial cells into fragments capable of stimulating monocytes to produce pro-inflammatory mediators, including IL-6 and IL-8. Their data provided evidence that platelets, beyond their classic role in hemostasis, contribute to the binding and catabolism of HA and can serve as an indirect provider of pro-inflammatory cytokines [77]. Therefore, understanding the mechanism by which platelets degrade HA is important in order to understand the role platelets play in the pathogenesis of inflammatory disease, including IBD.
CHAPTER II

PLATELET HYALURONIDASE-2: AN ENZYME THAT TRANSLOCATES TO THE SURFACE UPON ACTIVATION TO FUNCTION IN EXTRACELLULAR MATRIX DEGRADATION.

2.1. ABSTRACT

Following injury, platelets rapidly interact with the exposed extracellular matrix (ECM) of the vessel wall and the surrounding tissues. Hyaluronan (HA) is a major glycosaminoglycan component of the ECM and plays a significant role in regulating inflammation. We have recently reported that human platelets degrade HA from the surfaces of activated endothelial cells into fragments capable of inducing immune responses by monocytes. We also showed that human platelets contain the enzyme hyaluronidase-2 (HYAL2), one of two major hyaluronidases that digest HA in somatic tissues. The deposition of HA increases in the inflamed tissues in several inflammatory diseases, including Inflammatory Bowel Disease (IBD). We therefore wanted to define the mechanism by which platelets degrade HA in the inflamed tissues. In this study, we show that human platelets degrade the pro-inflammatory matrix HA through the activity of HYAL2 and that platelet activation causes the immediate translocation of HYAL2. 
from a distinct population of α-granules to platelet surfaces, where it exerts its catalytic activity. Finally, we show evidence that patients with IBD have lower platelet HYAL2 levels and activity than healthy controls.

2.2. INTRODUCTION

Hyaluronan (HA) is a ubiquitous glycosaminoglycan and a major component of the extracellular matrix (ECM), and it has a crucial role in regulating inflammation [78]. HA is produced by the hyaluronan synthase enzymes (HAS1-3) and is composed of repeating disaccharides of D-glucuronic acid and N-acetylglucosamine. Not only can HA be synthesized and released, but it can also form a voluminous pericellular coat that surrounds cells. The HA coat is either anchored to the cell surface through binding to specific cell-surface receptors, such as CD44, or can be retained at the cell surface by sustained transmembrane interactions with its synthases [79]. Interestingly, a growing body of literature suggests that different sizes of HA exert a wide spectrum of functions [38]. In tissues under normal conditions, HA is present in its high molecular weight (HMWHA) form (1-10X10^6 Da). HMWHA functions as a structural, hydrating polymer and is also known to be anti-inflammatory [44], protecting from T-cell-mediated liver injury and bleomycin-mediated lung injury in mice [45,46]. HMWHA also promotes the suppressive effects of regulatory CD4^+ CD25^+ T cells [47].

Increased HA deposition has also been reported in many inflammatory diseases including Inflammatory Bowel Disease (IBD), arthritis, and asthma [42,43]. Importantly, degradation of HA results in HA fragments that function as Damage Associated Molecular Patterns (DAMPs) [80]. Fragmented HA contributes to wound healing,
angiogenesis and inflammation and is capable of signaling cellular responses through specific receptors [37]. For example, HA fragments can activate macrophages and dendritic cells and can stimulate the transcription of inflammatory genes including TNF-α, IL-12, and IL-1b [44]. In monocytes, HA fragments were able to stimulate the production of IL-6 and monocyte chemoattractant protein-1 through CD44 and toll-like receptor 4 (TLR4) [49]. Recent studies have shown that HA fragments can also induce innate host defense responses at the intestinal epithelium by promoting the expression of human β-defensin-2, a potent antimicrobial peptide [50].

The accumulation of HA fragments in tissues during injury or under inflammation is thought to be largely the result of enzymatic degradation of HA, mainly through HYAL1 and HYAL2 in somatic tissues [18,37]. HYAL2 is a GPI-anchored protein that digests HA with the cooperation of CD44, the classical HA receptor [81]. Digested HA is then either internalized into the lysosome for further digestion, or is released into the environment [77,81]. KIAA1199 has recently been identified as a HA binding protein and a contributor to HA degradation [30]. HA digestion can also occur by non-enzymatic mechanisms, mainly through reactive oxygen species [35].

We previously reported that mouse and human platelets and their megakaryocytic precursors express both HYAL2 mRNA and protein with no evidence for HYAL1. We also showed that platelets can degrade endothelial pro-inflammatory matrix HA into signaling sized HA fragments that stimulate monocytes [77]. Platelets can act as inflammatory cells, beyond their main role in thrombus formation [59]. They store numerous inflammatory mediators in their granules, which can be released upon activation [64]. For example, CD154 on the surface of activated platelets can promote
leukocyte recruitment to sites of inflammation [82]. Platelets also express a number of the TLR family members. Platelet TLR4, for instance, can bind bacterial LPS; whereas TLR9 expressed on activated platelets can mediate bacterial DNA sequestration [70,83].

In this report, we define a novel mechanism by which platelets modify the ECM. We demonstrate that after activation, platelets degrade the pro-inflammatory matrix using HYAL2. This activation-dependent mechanism results in the immediate translocation of HYAL2 from α-granules to platelet surfaces, where it exerts its catalytic activity. Furthermore, we show that platelets of patients with IBD have lower HYAL2 protein content and hyaluronidase activity than control platelets, which might explain the accumulation of the leukocyte-adhesive HA observed in IBD tissues.

2.3. MATERIALS AND METHODS

2.3.1. Materials

Fetal Bovine Serum (FBS) was from GIBCO Life Technologies, Carlsbad, CA. 100 µM cell screens were from EMD Millipore, Germany. Acid-Citrate-Dextrose (ACD) was from BD Biosciences, Franklin Lakes, NJ. Streptomyces hyaluronidase, Benzonase® and prostaglandin E1 were from Sigma Aldrich, St. Louis, MO. Thrombin Receptor Activating Peptide (TRAP-6) was from Tocris Bioscience, UK. Anion exchange spin columns were from Thermo Scientific, Waltham, MA. Hyaluronan ELISA-like assay was from Echelon Biosciences, Salt Lake City, UT. Select-HA™ was from Lifecore Biomedical, LLC, Chaska, MN. Vectasheild Mounting Medium with DAPI was from Vector Labs, Burlingame, CA.
2.3.2. Cell Isolation and Culture

Human intestinal mucosal smooth muscle cells (M-SMCs) were isolated from colon surgical specimens (obtained from the Department of Surgical Pathology at the Cleveland Clinic) and cultured as previously described [51]. Briefly, 3-centimeter strips of the colon mucosal layer were incubated in Hanks’ BSS (HBSS) containing 0.15% dithiothreitol (w/v) for 30 min, washed in HBSS containing 1 mM EDTA for 3 h, and finally washed in HBSS for 2 h. The strips were cut into small pieces and then digested with 0.1 mg/mL collagenase and 0.1 mg/mL DNase for 18 h. The liberated cells were filtered through 100 µM cell screens, washed, suspended in DMEM/F12 media containing 10% FBS and cultured on tissue culture plastic incubated at 37°C in a 5%CO₂ humidified environment.

2.3.3. Platelet Isolation

Blood was collected by sterile venipuncture (without use of a tourniquet) directly into tubes containing Acid-Citrate-Dextrose (ACD) from consented healthy donors as approved by the Cleveland Clinic Institutional Review Board. The citrated blood was centrifuged for 20 min at 200g without applying break. The Platelet-Rich Plasma (PRP) layer was then collected, prostaglandin E1 was added to a final concentration of 35 ng/ml, and the PRP centrifuged for 20 min at 600g. The resulting platelet pellet was then washed twice with HBSS containing 35 ng/mL PGE1.

2.3.4 Cell co-culture assay for platelet hyaluronidase activity and fragment generation
Confluent human intestinal mucosal smooth muscle cells (M-SMCs) isolated from colon surgical specimens (obtained from the Department of Surgical Pathology at the Cleveland Clinic) were grown in 24-well cell culture plates and treated without or with 50 μg/mL polyI:C for 18 h. Culture media were replaced with RPMI media containing 1% FBS alone or containing freshly isolated resting platelets or Thrombin Receptor Activating Peptide (TRAP-6)-activated platelets (25 μM for 1 min at RT). Co-cultures were incubated for 2 h at 37°C. In some experiments, platelets were pre-incubated with blocking antibody against HYAL2 (1, 12, and 25 μg/ml) (Thermo) or control IgG (25 μg/ml). Culture media were collected and centrifuged to remove platelets. Total HA released into the media was measured using a competitive ELISA-like assay (Echelon) according to the manufacturer’s protocol. Unbound platelets, obtained by collection and centrifugation of culture media, and SMC-bound platelets, obtained by scraping SMCs from wells, were analyzed by flow cytometry for their surface P-selectin and HYAL2 expression.

2.3.5. Platelet microparticle isolation

Platelet microparticles were isolated as previously described [84]. Briefly, freshly isolated platelets were incubated without or with 15 μM TRAP for 10 minutes at 37°C and centrifuged at 750g for 20 minutes (platelet fraction). The supernatant platelet releasate was placed on ice and centrifuged at 10,000g for 30 minutes at 4°C to obtain the microparticles fraction. Platelet fraction (750g pellet), microparticles’ fraction (10^4 g pellet), and platelet releasate (10^4 g supernatant) were analyzed by western blotting for the presence of HYAL2.
2.3.6. Immunohistochemical staining of platelets

Resting or TRAP-activated freshly isolated platelets were fixed in 3.5% paraformaldehyde for 30 min at RT. Fixed platelets were spun onto a poly-l-lysine coated 8-well chamber slide at 250g for 10 min. Where indicated, permeabilization of platelets was done by adding cold methanol for 5 min. Fixed specimens were incubated with blocking buffer (2% FBS in HBSS) for 1 h. Non-permeabilized platelets were incubated with antibodies against HYAL2 and P-selectin. Permeabilized specimens were incubated with an antibody against HYAL2 and an antibody against one of the following: CD42b, LAMP2, PDI, KDEL, LAMP-1, NEU1, P-selectin, vWF, and fibrinogen. A permeabilized specimen was incubated with antibodies against P-selectin and vWF as a positive control for co-localization. After washing, specimens were incubated with the appropriate Alexa Fluor® secondary antibody for 45 min at RT, washed again, and Vectasheild Mounting Medium with DAPI was added. Images were obtained using either Leica TCS SP5 II Confocal/Multi-Photon high speed upright microscope or Leica upright microscope DM5500 B (Leica, Germany). Pearson’s correlation coefficients were obtained by analyzing individual images of the Z-stack using Image-Pro Plus software (Rockville, MD). See table 2.1 for a complete list of antibodies used and concentrations.
Table 2.1: List of antibodies used for the immunohistochemical staining of platelets.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host species</th>
<th>Company</th>
<th>Concentration used</th>
<th>Secondary antibody used*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYAL2</td>
<td>Rabbit polyclonal</td>
<td>Thermo Scientific (Waltham, MA)</td>
<td>1:50</td>
<td>Alexa Fluor® 488 Donkey Anti-Rabbit IgG (1:1000)</td>
</tr>
<tr>
<td>CD42b</td>
<td>Goat polyclonal</td>
<td>Santa Cruz Biotech (Santa Cruz, CA)</td>
<td>1:100</td>
<td>Alexa Fluor® 568 Donkey Anti-Goat IgG (1:1000)</td>
</tr>
<tr>
<td>LAMP1</td>
<td>Mouse monoclonal</td>
<td>Ebioscience (San Diego, CA)</td>
<td>1:50</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
</tr>
<tr>
<td>LAMP2</td>
<td>Mouse monoclonal</td>
<td>Ebioscience (San Diego, CA)</td>
<td>1:50</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
</tr>
<tr>
<td>PDI</td>
<td>Mouse monoclonal</td>
<td>Abcam (Cambridge, England)</td>
<td>1:100</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
</tr>
<tr>
<td>KDEL</td>
<td>Mouse monoclonal</td>
<td>Abcam (Cambridge, England)</td>
<td>1:100</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
</tr>
<tr>
<td>vWF</td>
<td>Goat polyclonal</td>
<td>Santa Cruz Biotech (Santa Cruz, CA)</td>
<td>1:25</td>
<td>Alexa Fluor® 568 Donkey Anti-Goat IgG (1:1000)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Goat polyclonal</td>
<td>Santa Cruz Biotech (Santa Cruz, CA)</td>
<td>1:50</td>
<td>Alexa Fluor® 568 Donkey Anti-Goat IgG (1:1000)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Mouse monoclonal</td>
<td>Cedarlane Labs (Burlington, ON)</td>
<td>1:100</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
</tr>
<tr>
<td>NEU1</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz Biotech (Santa Cruz, CA)</td>
<td>1:50</td>
<td>Alexa Fluor® 568 Goat Anti-Mouse IgG (1:1000)</td>
</tr>
</tbody>
</table>
2.3.7 Flow Cytometry

Freshly isolated resting or TRAP-activated (25 μM for 1 min at RT) platelets were fixed in 1% paraformaldehyde for 2 h at RT. Fixed platelets were washed then incubated for 1 h at RT in flow cytometry buffer (0.1% BSA, 0.05% sodium azide in PBS) with anti-HYAL2 (Thermo) (1:100), Allophycoerythrin (APC)-conjugated anti-P-selectin (Ebioscience) (1:1000), and anti-CD16/CD32 (to block the Fc receptors, 1:100). Platelets were washed then incubated with Alexa Fluor® 568 Goat Anti-Rabbit IgG for 30 min at RT. 30,000 events/sample were acquired on a BD Bioscience LSR II (BD Biosciences) flow cytometer. Data were analyzed using FlowJo (Tree Star, Ashaland, OR).

2.3.8 Subcellular fractionation of platelets using differential centrifugation

Freshly isolated platelets were sonicated and subcellular fractionation was achieved as previously described [85]. Briefly, platelet lysates were centrifuged at 1,000g to remove unlysed platelets, and then at 19,000g to obtain pellets that contain granules and lysosomes. Supernatants were ultracentrifuged at 100,000g to obtain pellets containing membranes and supernatants containing cytosol. Membranes were further fractionated into dense tubular system (DTS) and plasma membranes by layering on 40% sucrose and ultracentrifuging at 100,000g. Fractions containing equal total proteins were analyzed by immunoblotting for the presence of HYAL2 and CD42b.

2.3.9 Immuno-electron microscopy
Freshly isolated resting or TRAP-activated platelets were fixed with 3.5% paraformaldehyde and 0.09% gluteraldehyde for 30 min at RT and then centrifuged. Platelet pellets were washed with PBS, dehydrated with ethanol (30%-100%), embedded with LR White medium, and polymerized at 50°C for 48 h. Ultrathin sections cut with a diamond knife (87 nm) were mounted on formvar-coated nickel grids. Grids were rehydrated with PBS, blocked with 1% BSA for 30 min, and incubated with rabbit anti-HYAL2 antibody (Abcam) (1:50) for 18 h. Grids were washed and incubated with 10 nm gold-conjugated goat anti-rabbit IgG (Redding, CA) (1:10) for 1 h. Grids were washed again, fixed with 1% gluteraldehyde, and washed with distilled water. Grids were then stained with uranyl acetate and lead citrate, dried and examined with a FEI Tecnai G2 Spirit BioTWIN Transmission Electron Microscope (FEI Company, Hillsboro, OR) at 60 kV.

2.3.10 Statistical analysis

All statistics were performed using the Student t test. Data is presented as mean ± standard error unless otherwise mentioned. Patient data were presented as scatter plot showing mean ± standard error and as box-and-whiskers plot showing median and 10-90 percentiles. P-values below 0.05 were considered statistically significant.

2.3.11. Immunofluorescence histochemical staining of M-SMCs

M-SMCs from the co-culture assay grown on coverslips were washed and then fixed in cold methanol for 5 min. Coverslips were incubated in blocking buffer (2% FBS in HBSS) for 1 h at RT, and then in blocking buffer containing 5 µg/mL biotinylated HA binding protein (EMD Millipore, Germany) and anti-CD42b (1:100) for 18 h at 4°C.
Coverslips were then washed and incubated with blocking buffer containing detection reagents Alexa Fluor® 488 streptavidin (1:500) and Alexa Fluor® 568 Donkey Anti-Goat IgG (1:1000) (Life Technologies, Carlsbad, CA) for 45 min at RT. The stained coverslips were washed and mounted onto slides. Images were obtained using Leica TCS SP5 II Confocal or Leica upright microscope DM5500 B (Leica, Germany).

2.3.12 HA isolation and sizing

HA was purified from stimulated M-SMCs before and after they were co-incubated with platelets. M-SMCs were incubated with 0.5 mg/ml proteinase-K in PBS for 18 h at 60°C, then with benzonase® (50 U/ml) for 1 h at 37°C. Samples were then concentrated using speedvac and dialyzed against 0.1 M NaCl. Samples were applied to anion exchange spin columns (Thermo) and centrifuged for 5 min. Columns were washed with 0.1 M and 0.4 M NaCl before HA was eluted with 0.8 M NaCl. Eluted samples containing HA were dialyzed against water, concentrated, and analyzed using 0.5% agarose sizing gel as described previously [86]. Stained bands in the gel were confirmed to be HA by demonstrating sensitivity to Streptomyces hyaluronidase (2 U/ml for 1 h at 37°C).

2.3.13 Hyaluronidase assay using immobilized purified HA

Freshly isolated resting, or TRAP-activated, or lysed platelets resuspended in neutral or acidic RPMI medium were added to a 96-well plate coated with HA (Echelon) (3X10⁸ platelets/well). The plate was incubated at 37°C for 18 h and then washed. Residual HA was detected colorimetrically using HA detection system provided by the
manufacturer. *Streptomyces* hyaluronidase (2 U/ml) was used as a positive control and for defining maximum activity.

### 2.3.14 Hyaluronidase assay using purified HA in solution

Platelets were incubated at 37°C for 18 hours in RPMI medium containing 1000 kDa Select-HA™ (5 µg/ml), an enzymatically synthesized commercial HA with high size control. Samples from the incubation were analyzed for HA by 0.5% agarose gel electrophoresis as described previously (0.5 µg of HA/lane).

### 2.3.15 Subcellular fractionation of platelets using sucrose density gradient

This was achieved as previously described [87]. Briefly, freshly isolated platelets were sonicated and applied on top of a linear 30-60% sucrose gradient followed by ultracentrifugation for 100,000g for 90 min. Nine fractions were collected by pipetting from the top, and equal volumes of fractions were analyzed by immunoblotting for the presence of HYAL2, P-selectin, von Willbrand factor (vWF), CD42b, and LAMP-1.

### 2.4. RESULTS

#### 2.4.1 Platelets degrade pro-inflammatory matrix HA

de la Motte et al. have recently demonstrated that platelets can digest HA from surfaces of activated endothelium, which produces fragmented HA capable of promoting cellular responses [77]. In order to investigate the mechanism by which platelets cleave matrix HA, we adopted a cell co-culture assay. Cultured human intestinal mucosal smooth muscle cells (M-SMCs) isolated from surgical specimens were treated with polyI:C, a double stranded RNA used to mimic a virus infection, to stimulate production
of pro-inflammatory, leukocyte-adhesive HA. Freshly isolated human platelets were co-incubated with polyI:C stimulated M-SMCs for two hours at 37°C. Cell-associated HA was then purified and analyzed by agarose gel electrophoresis. The highly polydisperse cell-associated HA in the size range of 0.5-2 x 10^6 Da was detected on the stimulated cells (figure 2.1A lane 1). However, HMWHA was not detected on cells co-incubated with platelets (figure 2.1A lane 2) indicating that platelets cleared most of the pro-inflammatory matrix HA on the M-SMC surface. Replicate samples were subjected to Streptomyces hyaluronidase, an enzyme that specifically degrades HA, to confirm that the stained bands were HA (figure 2.1A lane 3&4). In order to test whether the HA cleaved by platelets is released into the culture medium, we measured HA in the culture fluid by an ELISA-like assay. As expected, polyI:C treated cells released significantly higher amounts of HA than non-treated cells (P<0.001). However, when polyI:C treated cells were incubated with platelets, the amount of HA released was significantly increased (P<0.001) (Figure 2.1B). The effect that platelets have on HA in the extracellular matrix is striking when observed by histochemical staining. Upon polyI:C treatment, M-SMCs produced large amounts of leukocyte-adhesive HA structures or “HA cables” (figure 2.1C-green). Following co-incubation with platelets, HA cables were completely removed from the surfaces of M-SMC, confirming the role of platelets in clearing pro-inflammatory HA. To assess the activation state of platelets in culture and whether culture conditions (e.g. serum and SMC surface molecules) contribute to platelet activation, we analyzed their surface P-selectin by flow cytometry. We found that platelets that were bound to SMCs demonstrated significantly higher surface expression
of P-selectin compared to unbound platelets. Importantly, bound platelets also showed a significant increase in surface HYAL2 compared to unbound platelet (figure 2.1D).
Figure 2.1: Platelets digest HA in the inflammatory matrix. PolyI:C stimulated human M-SMCs were co-incubated with freshly isolated human platelets for 2 hours at 37°. (A) Agarose gel electrophoretic analysis of HA purified from polyI:C stimulated M-SMCs before (lane 1) and after (lane 2) co-incubation with platelets. Polydisperse HA with a size range of 0.5-2 X10^6 Da associated with M-SMCs was no longer detected after co-incubation with platelets. Replicate samples were treated with *Streptomyces* hyaluronidase (HAase), an enzyme that specifically digests HA, to confirm that the stained bands were HA (lanes 3&4). HA ladder units: 10^6 Dalton.
Figure 2.1 (cont.): Platelets digest HA in the inflammatory matrix. PolyI:C stimulated human M-SMCs were co-incubated with freshly isolated human platelets for 2 hours at 37°. (B) ELISA-like assay measurement of HA released into the media by M-SMCs. Co-incubation of M-SMCs with platelets results in a significant increase of HA in culture media. (Data represent mean ± SE of 10 separate experiments, ***P<.001.)
Figure 2.1 (cont.): Platelets digest HA in the inflammatory matrix. PolyI:C stimulated human M-SMCs were co-incubated with freshly isolated human platelets for 2 hours at 37°. (C) Histochemical staining of M-SMC-associated HA (green). M-SMCs were fixed in cold methanol, and HA was detected with biotinylated HA binding protein and Alexa Fluor® 488-conjugated streptavidin. M-SMCs responded to polyI:C treatment by producing high amounts of HA. The co-incubation of platelets with polyI:C stimulated M-SMCs caused the removal of the M-SMC surface-associated HA. Image details for figure 1C: Microscope: Leica upright microscope DM5500 B (Leica, Germany). Objective: HC PLAN APO 20X/0.7NA dry. Camera: QImaging Retiga cooled CCD camera. Acquisition software: QCapture Suite Software (QImaging, Surrey, BC). Scale bar: 100 µm.
Figure 2.1 (cont.): Platelets digest HA in the inflammatory matrix. PolyI:C stimulated human M-SMCs were co-incubated with freshly isolated human platelets for 2 hours at 37°. (D) Fold-increase in surface P-selectin and HYAL2 MFI of SMC-bound platelets in comparison to unbound platelets following culture as measured by flow cytometry. SMC-bound platelets demonstrated significantly higher surface P-selectin and HYAL2 compared to unbound platelets after culture.
2.4.2 Degradation of matrix HA by platelets is HYAL2 dependent

The two somatically active HA degrading enzymes in humans are HYAL1 and HYAL2 [27]. We showed previously that platelets contain only HYAL2 protein and mRNA, with no evidence of HYAL1 [77]. Additionally, we observed in the previous experiments that HYAL2 increases on the surface of platelets bound to SMCs in culture. Therefore, we hypothesized that pro-inflammatory HA clearance by platelets is mediated by platelet HYAL2. To test this hypothesis, we incubated platelets with different concentrations of HYAL2 neutralizing antibodies, or with non-specific control IgG, before they were co-incubated with polyI:C-stimulated M-SMCs. We then analyzed HA released into the media by the ELISA-like assay. HA concentrations in media collected from M-SMCs that were incubated with HYAL2 antibody-treated platelets were significantly lower than media collected from cells incubated with either untreated platelets or platelets pre-incubated with IgG (Figure 2.2A) ($P<0.05$, N=3). We confirmed that the effect of the blocking antibody is specific to platelet HYAL2, not SMC HYAL2, by centrifuging the HYAL2 antibody-treated platelets to remove excess antibody before they were added to the culture (data not shown). To further confirm HYAL2 antibody blockade, we performed histochemical staining of HA on polyI:C stimulated M-SMC (green) co-incubated with platelets (red for CD42b) in the absence or presence of HYAL2 antibody (Figure 2.2B). HA cables on the surfaces of M-SMCs that were co-incubated with HYAL2 antibody-treated platelets remained intact whereas HA on M-SMCs co-incubated with untreated platelets was removed, consistent with our ELISA-like data. Interestingly, HYAL2 antibody-treated platelets retained their HA cable
binding ability, an observation previously reported when monocytes or platelets were incubated with HA cables at 4°C.
Figure 2.2: Platelets degrade pro-inflammatory matrix HA using HYAL2. PolyI:C stimulated human M-SMCs were co-incubated with untreated freshly isolated human platelets or with HYAL2 blocking antibody-treated platelets for 2 hours at 37°. (A) ELISA-like assay measurement of the amounts of HA released into the media by M-SMCs. Co-incubation of stimulated M-SMCs with HYAL2 antibody-treated platelets resulted in a significant decrease in HA released into media compared to untreated or IgG-treated platelets (N=3, *P<.05). The inhibiting effect of the HYAL2 blocking antibody was dose dependent.
Figure 2.2 (cont.): Platelets degrade pro-inflammatory matrix HA using HYAL2. PolyI:C stimulated human M-SMCs were co-incubated with untreated freshly isolated human platelets or with HYAL2 blocking antibody-treated platelets for 2 hours at 37°.

(B) Histochemical staining of M-SMC-associated HA (green) and platelets (red = CD42b) in the absence or presence of HYAL2 antibody. Whereas untreated platelets degraded HA on stimulated M-SMCs, HYAL2 antibody-treated platelets bound HA cables on the surfaces of M-SMCs without degrading them. Image details for figure 2B:
Microscope: Leica TCS SP5 II Confocal/Multi-Photon high speed upright microscope (Leica, Germany). Objective: HCX PL APO 40X/1.25NA oil immersion. Detector: Leica HyD™ system. Acquisition software: Leica LAS AF software (Leica, Germany). Scale bar: 25 µm.
2.4.3 Degradation of purified HA by platelets and platelet lysates only occurs at low pH

HYAL2 is reported to be an acid active enzyme [23]. With the observation that platelets degrade matrix HA by HYAL2 under physiological conditions (pH 7.2), we tested whether platelets have the ability to degrade purified HA under neutral pH using two approaches: 1) can platelets degrade purified HA immobilized to a surface? 2) can platelets degrade purified HA in solution? We first used a range of pH conditions. Platelets that were incubated with immobilized purified HA at neutral pH displayed no HA degrading activity; whereas platelets incubated under acidic conditions (pH 2.5-4.5) demonstrated HAase activity. Platelet lysates also had a peak HAase activity at pH 3.5 with no measurable activity at neutral pH (Figure 2.3A). Surprisingly, platelet lysates showed significantly higher HAase activity (60% maximum) than whole platelets (20% maximum) ($P<0.001$, $N=3$) (figure 2.3B). Maximum activity was achieved by *Streptomyces* hyaluronidase. The data suggest that the majority of the resting platelet HYAL2 is not on the surface. To test whether platelets can degrade purified HA in solution, platelets were incubated with Select-HA™ (1000 kDa), a commercial monodisperse HA prepared through enzymatic synthesis where a high level of size control is possible. As observed with immobilized HA, platelets were only able to digest purified HA in solution under acidic pH (Figure 2.3C).
Figure 2.3: Platelets and platelet lysates can only degrade purified HA under acidic pH. (A&B) Platelets and platelet lysates were incubated with purified HA immobilized to wells of a 96-well plate for 18 hours at 37°C (3X10^8 platelets/well). At the end of the incubation, platelets and degraded HA were washed away and HAase activity was measured by detecting residual HA colorimetrically. Maximum activity (100%) was achieved by *Streptomyces* hyaluronidase. (A) HAase activity of platelets and their lysates at different pHs. Platelets and their lysates demonstrated purified HA-degrading activity only under acidic pH (optimum pH=3.5) with no detectable activity at neutral pH.
Figure 2.3 (cont.): Platelets and platelet lysates can only degrade purified HA under acidic pH. (A&B) Platelets and platelet lysates were incubated with purified HA immobilized to wells of a 96-well plate for 18 hours at 37°C (3X10^8 platelets/well). At the end of the incubation, platelets and degraded HA were washed away and HAase activity was measured by detecting residual HA colorimetrically. Maximum activity was achieved by *Streptomyces* hyaluronidase. (B) Purified HA-degrading activity of platelets compared to the activity of their lysates. Platelet lysates have significantly higher purified HA-degrading activity than intact platelets. (***(P<.001, N=3).
Figure 2.3 (cont.): Platelets and platelet lysates can only degrade purified HA under acidic pH. (C) Purified HA (Select-HA™, 1000 kDa) in RPMI medium (5 mg/ml) was incubated with platelets (300 x 10^6 plts/ml) for 18 hours at 37°C. Platelets degraded purified HA only under acidic pH (pH=3.5), but not under neutral pH.
2.4.4 HYAL2 becomes surface-expressed on thrombin receptor-activated platelets.

The observations that HYAL2 increased on the surface of SMC-bound platelets, which was also accompanied by an increase in surface P-selectin expression, and that platelet lysates had significantly higher HAase activity than intact platelets suggested that platelet activation may have a role in the surface expression of HYAL2. Therefore, we analyzed the normal HYAL2 expression parameters in resting and activated platelets. First, we examined whether activated platelets package HYAL2 into microparticles. Immunoblot analysis revealed that, upon activation, HYAL2 remains associated with platelets, and does not associate with microparticles or any platelet releasate, even after 30 minutes of incubation with TRAP (figures 2.4A & 2.4B). We then compared HYAL2 expression in paraformaldehyde fixed, non-permeabilized platelets by immunohistochemical staining using fluorescence microscopy (Figure 2.4C). Surface expression of P-selectin (red) was used as a platelet activation marker. TRAP-activated platelets showed significantly higher HYAL2 staining levels than resting platelets. Since platelets were not permeabilized, the data provided evidence that HYAL2 is targeted to platelet surfaces only upon activation. Next we analyzed surface expression of HYAL2 in unstimulated and TRAP-stimulated platelets by flow cytometry. To eliminate the spontaneously activated platelets in the unstimulated population, we gated on the strictly P-selectin negative population in that sample. The P-selectin negative population demonstrated no surface HYAL2 expression, whereas the P-selectin positive population in the TRAP-stimulated sample showed significant surface HYAL2 expression (figure 2.4D). We also determined, using flow cytometry analysis, that only 6.8 ± 1.3% (±SE) of non-activated (P-selectin negative) platelets in the tested population expressed surface
HYAL2 compared to 37.6 ± 6.4% (±SE) of activated platelets ($P<0.001$, $N=9$) (Figure 2.4E). Flow cytometry analysis also showed a 3-fold increase in Mean Fluorescence Intensity (MFI) of surface HYAL2 in TRAP-activated platelets compared to non-activated platelets ($P<0.001$, $N=9$) (Figure 2.4F).
**Figure 2.4: HYAL2 becomes surface expressed upon thrombin receptor-mediated platelet activation.** (A) Freshly isolated human platelets were treated without or with TRAP (15 µM) for 10 minutes before microparticles were isolated. Immunoblot analysis showed that, upon activation, HYAL2 was associated with platelets (750 g pellet) and not with microparticles (10⁴ g pellet) or platelet releasate (10⁴ sup.). (B) Immunoblot analysis showing that HYAL2 remains associated with platelets (750g pellet) and not with microparticles or releasate (REL) even after 30 minutes incubation with TRAP.
Figure 2.4 (cont.): HYAL2 becomes surface expressed upon thrombin receptor-mediated platelet activation. (C) Freshly isolated human platelets were treated without or with TRAP (25 µM) for 1 minute before they were fixed with 3.5% paraformaldehyde for 30 minutes. Fixed platelets were spun down onto poly-l-lysine coated slides and stained for P-selectin (red) and HYAL2 (green). Similar to P-selectin, HYAL2 was only detected on the surfaces of activated platelets. Image details for figure 4A: Microscope: Leica upright microscope DM5500 B (Leica, Germany). Objective: HCX PLAN APO 63X/1.32NA oil immersion. Camera: QImaging Retiga cooled CCD camera. Acquisition software: QCapture Suite Software (QImaging, Surrey, BC). Scale bar: 20 µm.
Figure 2.4 (cont.): HYAL2 becomes surface expressed upon thrombin receptor-mediated platelet activation. (D,E,&F) Freshly isolated platelets were treated without or with TRAP (25 µM) for 1 min at RT before they were fixed in 1% paraformaldehyde for 2 hours at RT. Fixed platelets were stained for HYAL2 and P-selectin and analyzed using flow cytometry. (D) Representative histograms of surface P-selectin and HYAL2 staining in unstimulated and TRAP-stimulated platelets. The P-selectin negative population in unstimulated platelets demonstrated no surface HYAL2 expression, whereas the P-selectin positive population in the TRAP-stimulated sample showed significant surface HYAL2 expression.
Figure 2.4 (cont.): HYAL2 becomes surface expressed upon thrombin receptor-mediated platelet activation. (D,E,&F) Freshly isolated platelets were treated without or with TRAP (25 µM) for 1 min at RT before they were fixed in 1% paraformaldehyde for 2 hours at RT. Fixed platelets were stained for HYAL2 and P-selectin and analyzed using flow cytometry. (E) Percentage of surface HYAL2 positive platelets measured by flow cytometry. 37.6 ± 6.4% (±SE) of TRAP-activated platelets expressed surface HYAL2 compared to only 6.8 ± 1.3% (±SE) of non-activated (P-selectin negative) platelets (**P<0.001, N=9).
Figure 2.4 (cont.): HYAL2 becomes surface expressed upon thrombin receptor-mediated platelet activation. (D,E,&F) Freshly isolated platelets were treated without or with TRAP (25 µM) for 1 min at RT before they were fixed in 1% paraformaldehyde for 2 hours at RT. Fixed platelets were stained for HYAL2 and P-selectin and analyzed using flow cytometry. (F) Fold-increase in surface HYAL2 MFI of TRAP-activated platelets in comparison to non-activated (P-selectin negative) platelets. Activated platelets demonstrated 3-fold increase in surface HYAL2 expression compared to non-activated platelets (***P<.001, N=9).
2.4.5 Platelet HYAL2 is localized to a distinct population of α-granules

Since HYAL2 appears to be expressed on the platelet surface only upon activation, we hypothesized that HYAL2 is stored inside platelet granules in resting platelets. To test this, we wanted to determine whether HYAL2 partitions with granules, cytosol or membranes [plasma membranes and dense tubular system (DTS)] in resting platelets. First, we separated platelet granules (α-granules, dense granules, lysosomes and mitochondria) from platelet membranes by differential centrifugation. The platelet membrane marker CD42b was detected, as expected, in the plasma membrane fraction (Figure 2.5A). HYAL2, however, was detected specifically in the granule fraction. Next, to define the granule type in which HYAL2 is stored, sucrose density gradients were used to separate platelet subcellular compartments (Figure 2.5B). HYAL2 and P-selectin, an α-granule marker, were enriched in the same sucrose fraction, suggesting that HYAL2 is stored in platelet α-granules. CD42b and LAMP-1 were used as plasma membrane and lysosomal markers, respectively.

Subsequently, we analyzed co-localization of platelet HYAL2 with different platelet granule markers using fluorescence microscopy (Figure 2.5C). Consistent with the sucrose density gradient data, platelet HYAL2 staining did not co-localize with known specific markers of plasma membranes (CD42b), lysosomes (LAMP-1 and NEU1), dense granules (LAMP-2), T-granules (PDI), or the DTS (KDEL). Surprisingly, platelet HYAL2 also did not co-localize with the known specific markers of α-granules (vWF, P-selectin, and fibrinogen). The lack of co-localization between platelet HYAL2 and the α-granule markers we examined led us to conclude that HYAL2 is stored in a different subset of α-granules inside resting platelets. As a positive control, we analyzed
the co-localization of vWF and P-selectin, two known α-granule markers, in platelets. As expected, vWF and P-selectin showed significant levels of co-localization (Pearson correlation coefficient = 0.65). To finally determine that HYAL2 is stored in platelet α-granules, given the fact that it did not co-localize with known α-granule markers, we examined its localization by immuno-electron microscopy. Inside resting platelets, HYAL2 was clearly detected within α-granules, which were identified by their distinct morphology and number. However, whereas resting platelet surfaces appeared to be devoid of any HYAL2 expression, HYAL2 was clearly detected on the surfaces of TRAP-activated platelets (Figure 2.5D). Interestingly, HYAL2 appears to exist in clusters on platelet surfaces, unlike the previously reported uniform distribution of adhesion molecules, such as P-selectin [88].
Figure 2.5: HYAL2 is stored in a distinct subset of α-granules in resting platelets.

(A) Immunoblot analysis, for the presence of CD42b and HYAL2, of different platelet subcellular fractions obtained by differential centrifugation. HYAL2 was detected exclusively in the fraction containing granules, lysosomes and mitochondria (19,000g pellet); whereas CD42b was detected in the membranes fraction (100,000g pellet).
Figure 2.5 (cont.): HYAL2 is stored in a distinct subset of α-granules in resting platelets. (B) Platelets were sonicated and applied on top of a linear sucrose density gradient before the gradient was centrifuged at 100,000g for 90 min. Nine fractions were collected from top of the gradient. Equal volumes of fractions were analyzed by immunoblotting for the presence of CD42b (plasma membrane marker), LAMP-1 (lysosomal marker), P-selectin (α-granule marker), and HYAL2. HYAL2 and P-selectin were both enriched in fraction number 5, the α-granules fraction; whereas CD42b and LAMP-1 were enriched in fractions numbers 2 and 4, respectively.
Figure 2.5 (cont.): HYAL2 is stored in a distinct subset of α-granules in resting platelets. (C) Confocal microscopy images (maximum projection) of platelets stained for HYAL2 (green) and one of the following proteins: CD42b (plasma membrane marker), LAMP-2 (dense granule marker), PDI (T-granule marker), KDEL (dense tubular system marker), LAMP-1 and NEU1 (lysosomal markers), P-selectin (α-granule marker), vWF (α-granule marker), and fibrinogen (α-granule marker). HYAL2 did not co-localize with any of the tested markers. Pearson’s correlation coefficients were obtained by analyzing individual images (layers) of the Z-stack using Image-Pro Plus software (Rockville, MD). Image details for figure 5C: Microscope: Leica TCS SP5 II Confocal/Multi-Photon high speed upright microscope (Leica, Germany). Objective: HCX PL APO 63X/1.4NA oil immersion. Detector: Leica HyD™ system. Acquisition software: Leica LAS AF software (Leica, Germany). Scale bar: 1 µm.
Figure 2.5 (cont.): HYAL2 is stored in a distinct subset of α-granules in resting platelets. (D) Immuno-electron microscopy images showing resting and activated platelets. Washed human platelets were fixed, sectioned, and mounted on formvar-coated nickel grids. Ultrathin platelet sections were probed for HYAL2, and the bound antibody was labeled with immunogold (10 nm). In resting platelets, HYAL2 appeared to be localized within platelet α-granules (black arrows), whereas platelet surface appeared to be devoid of any HYAL2. However, in activated platelets, HYAL2 was clearly detected on the surface. Image details for figure 5D: Microscope: FEI Tecnai G2 Spirit BioTWIN Transmission Electron Microscope (FEI Company, Hillsboro, OR). Camera: Orius 832 CCD, 11 megapixel. Acquisition software: Digitalmicrograph (Gatan, Inc., Pleasanton, CA). Scale bars: 200 nm.
2.4.6 Activated platelets have higher HAase activity than non-activated platelets.

Platelet HYAL2 appeared on the surface of platelets after activation. Therefore, we postulated that platelet HAase activity also increases upon activation. We first examined whether activated platelets have higher HA degrading ability than non-activated platelets in the co-culture model. Replicate cultures of polyI:C treated M-SMCs were co-cultured with decreasing concentrations (300-10X10^6/well) of either TRAP-activated or non-activated freshly isolated platelets. The levels of HA released into the media by activated platelets were higher than HA released by non-treated platelets under all conditions, suggesting that activated platelets had higher HA degrading ability than non-activated platelets (Figure 2.6A). However, below platelet concentration of 30X10^6/well, non-treated platelets had no HA-degrading ability, whereas activated platelets retained significant levels of activity. We then examined whether activated platelets also have higher purified HA degrading activity compared to non-activated platelets. TRAP-activated and non-activated platelets were incubated with purified HA immobilized to wells of a 96-well plate (pH=3.5). Activated platelets demonstrated significantly higher HAase activity (80% maximum) than non-activated platelets (10% maximum) (P<0.001). Interestingly, activated platelets also showed significantly higher HAase activity than platelet lysates (Figure 2.6B). As expected, activated platelets also did not display purified-HA degrading ability under neutral pH (data not shown).
Figure 2.6: Thrombin receptor-mediated platelet activation results in increased platelet HAase activity. (A) PolyI:C stimulated human M-SMCs were co-incubated with decreasing concentrations of either untreated freshly isolated platelets or TRAP-activated platelets for 2 hours at 37°. The amounts of HA released into the media were measured by ELISA-like assay. Activated platelets caused a significantly higher increase in HA released into the media than non-activated platelets. At a platelet concentration of 30X10^6/well, non-activated platelets appeared to have no HAase activity compared to activated platelets, which still released significant amounts of HA into media. (**P<.01, N=3).
Figure 2.6 (cont.): Thrombin receptor-mediated platelet activation results in increased platelet HAase activity (B) Freshly isolated untreated or TRAP-activated or lysed platelets (3X10^8/well) were incubated with purified HA immobilized to wells of a 96-well plate for 18 h at 37°C (pH=3.5). Platelets and digested HA were then washed and remaining HA on the wells was detected colorimetrically. Activated platelets demonstrated significantly higher HAase activity (80% maximum) than non-activated platelets (10% maximum) and platelet lysate (50% maximum) (**P<.001, N=3). Maximum activity was achieved by Streptomyces hyaluronidase.
2.4.7 IBD patients have lower platelet HYAL2 protein and HAase activities than non-IBD controls

Multiple platelet abnormalities have been identified in patients with IBD, including increased numbers and levels of activation [75]. Also, pro-inflammatory HA is increased in the inflamed intestinal mucosa of IBD patients. We therefore hypothesized that platelet HYAL2 has a role in ECM-HA degradation in IBD and compared the levels of platelet HYAL2 and HAase activity in patients with IBD to non-IBD controls. The immunoblot assay and HAase assay we used were normalized to total protein because IBD patients are known to frequently have high platelet numbers and low platelet volume. We first analyzed HYAL2 levels in lysates of platelets from IBD patients (N=17) and non-IBD controls (N=13) and found that, interestingly, HYAL2 protein levels in IBD patient samples, on average, were 45% lower than samples from non-IBD controls ($P=0.01$) (Figure 2.7A). We then analyzed HAase activity in lysates of platelets from IBD patients (N=8) and non-IBD controls (N=8) and found that HAase activity in IBD patient samples was also significantly lower than samples from non-IBD controls ($P<0.001$) (Figure 2.7B).
Figure 2.7: Platelets from IBD patients have lower HYAL2 and HAase activity than their non-IBD counterparts. Platelets collected from IBD patients and from healthy controls, as approved by the Cleveland Clinic Institutional Review Board, were washed and lysed. Total protein concentrations of platelet lysates were determined using Bradford assay. (A) 25 µg of total protein from each sample was analyzed by immunoblotting for HYAL2. Densitometry analysis (ImageQuant TL, GE Healthcare Life Sciences, Fairfield, CT) showed that platelets from IBD patients (N=17) displayed an average reduction of 45% in HYAL2 protein levels compared to non-IBD controls (N=13) (*P=.01). Data are presented as both a scatter plot showing mean and a box-and-whiskers plot showing median of 10-90 percentiles.
Figure 2.7 (cont.): Platelets from IBD patients have lower HYAL2 and HAase activity than their non-IBD counterparts. Platelets collected from IBD patients and from healthy controls, as approved by the Cleveland Clinic Institutional Review Board, were washed and lysed. Total protein concentration of platelet lysates was determined using Bradford assay. (B) Platelet lysates containing 400 µg total protein were analyzed for their HAase activity. Platelet lysates were incubated with purified HA immobilized to wells of a 96-well plate for 18 h at 37°C (pH=3.5). Lysates and digested HA were then washed and remaining HA on the wells was detected colorimetrically. IBD platelets demonstrated significantly lower HAase activity (N=8) compared to their healthy counterparts (N=8) (**P<.001). Data are presented as both a scatter plot showing mean and a box-and-whiskers plot showing median of 10-90 percentiles.
2.5. CONCLUSION

In this report we show that platelets degrade HA from the surfaces of activated human intestinal M-SMCs under experimental physiological pH and that this degradation can be inhibited by blocking the activity of platelet HYAL2. We also demonstrate that platelet degradation of purified HA only occurs under acidic pH, consistent with previous reports on HYAL2 activity [92]. Although HYAL2 is known to be a cell surface protein [93], we found little evidence of it on the surfaces of resting platelets. Instead, HYAL2 was detected exclusively on the surfaces of activated platelets. We also show for the first time that HYAL2 is packaged within α-granules in resting platelets, and that upon activation, HYAL2 is translocated from α-granules to the platelet surface, where it functions to digest HA. Finally, we show evidence that patients with IBD have lower platelet HYAL2 contents and activity than healthy controls.
CHAPTER III

THE ROLE OF HYALURONAN BINDING PROTEINS AND PLATELET ACTIVATION IN PLATELET-HYALURONAN BINDING

3.1. ABSTRACT

Human platelets have the ability to degrade hyaluronan (HA) in the pro-inflammatory matrix through the activity of HYAL2, which is a GPI-anchored protein and one of two enzymes that degrade HA in somatic tissues. Platelet activation plays an important role in the expression of HYAL2. Platelet activation causes the immediate translocation of HYAL2 from a distinct population of α-granules to platelet surfaces, where it exerts its catalytic activity. Importantly, patients with inflammatory bowel disease (IBD) have lower platelet HYAL2 levels and activity than healthy controls. However, in spite of the facts that platelets cleave HA from surfaces of endothelial and smooth muscle cells under cell-culture conditions and purified HA under acidic conditions, platelets still cannot degrade purified HA under neutral conditions. We hypothesized that HA-binding proteins and platelet activation have a role in facilitating the interaction between HA and platelets under experimental neutral conditions. In this study, we provide evidence that 1) platelets have the ability to degrade HA released from
polyI:C-stimulated cells, in solution; 2) platelet activation results in increased binding of platelets to HA; and 3) platelets express the newly discovered HA-binding protein KIAA1199 and release it upon activation. In some inflammatory diseases, like IBD, HA becomes associated with proteins in the form of cables; in addition, platelets circulate in a highly activated state. For those reasons, our data suggest that HA becomes a better substrate for platelets degradation under inflammatory conditions.

3.2. INTRODUCTION

HA is a non-sulfated, unbranched, glycosaminoglycan and a major component of the extracellular matrix. It is present generally in high amounts in many tissues and fluids, including the eye vitreous, the umbilical cord, amniotic fluid, and the joints. High levels of HA are also present in proliferating tissues and tissues undergoing repair. The turnover of HA occurs in a very fast rate in the body. The rapid catabolism of HA, through the activity of the hyaluronidase (HYAL) enzymes, represents a major mechanism by which HA levels are regulated in the body. HA catabolism is known to be dependent upon the classical HA binding receptor CD44 and involves mainly the two hyaluronidases: HYAL1 and HYAL2 [18, 27-29].

Studies have shown that the degradation of HA by HYAL2 is CD44-dependent. In human breast tumor cell line, the binding of HA to CD44 activates Rho kinase, which, in turn, causes the phosphorylation, and activation, of \( \text{Na}^+\text{-H}^+ \) exchanger (NHE1). The activity of NHE1 promotes acidification and creates an acidic extracellular matrix environment. The acidic environment provides a condition for HYAL2 to degrade HA at the cell surface. In a human embryonic kidney cell line, HYAL1 cannot degrade HA in
cells lacking CD44 and that its activity in cells expressing CD44 is primarily intracellular. Conversely, the activity of HYAL2 is strictly extracellular but also dependent upon CD44, which exists on the cell surface along with HYAL2 [28,29].

The current accepted model for HA degradation in the somatic tissues of vertebrates involves four enzymes: HYAL1, HYAL2, and two β-exoglycosidases. Degradation of HA starts at the cell surface. CD44 binds HA extracellularly and mediates the process by creating an acidic microenvironment. Then, the acid-active enzyme HYAL2 degrades large HA polymers into approximately 20-kDa polymers. These HA products get internalized into the lysosomes, where they get degraded by HYAL1 into tetra- and hexasaccharides before N-acetyl glucosaminidase and glucuronidase enzymes further degrade them into monosaccharides [18].

Our group has recently shown that platelets contain HYAL2. We demonstrated that after activation, platelets degrade HA in the pro-inflammatory matrix using HYAL2. This activation-dependent mechanism results in the immediate translocation of HYAL2 from α-granules to platelet surfaces, where it exerts its catalytic activity. Importantly, we showed that although platelets can cleave HA from surfaces of endothelial and smooth muscle cells under cell-culture conditions and purified HA under acidic conditions, they cannot degrade purified HA under neutral conditions [89].

CD44 belongs to a family of glycoproteins that are expressed on the surfaces of many cell types, mainly lymphocytes, macrophages, and epithelial cells. In 1994, Koshiishi et al. have demonstrated that CD44 is present on mouse megakaryocytes and peripheral blood platelets [90]. However, no evidence provided by this group, or any
other group, that human platelets express CD44. Our group also was not able to detect any CD44 levels in platelets by immunoblotting or immunofluorescence microscopy (data not shown). Therefore, HA degradation by platelets under neutral conditions cannot be explained by the presence of CD44, and investigating new platelet-HA binding mechanisms that do not involve CD44 is crucial to understanding the mechanism by which platelets cleave HA.

In this study, we provide evidence that platelets have the ability to degrade HA released from polyI:C-stimulated cells and that platelet activation results in increased binding of platelets to HA. Additionally, we show for the first time that platelets express the newly discovered HA-binding protein KIAA1199 and release it upon activation. In some inflammatory diseases, like IBD, HA becomes associated with proteins to form pathological HA structures known as HA cables [51]; in addition, platelets circulate in a highly activated state [75]. For those reasons, our data suggest that HA becomes a better substrate for platelets degradation under inflammatory conditions.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Fetal Bovine Serum (FBS) from GIBCO Life Technologies, Carlsbad, CA. 100 µM cell screens were from EMD Millipore, Germany. Acid-Citrate-Dextrose (ACD) was from BD Biosciences, Franklin Lakes, NJ. Streptomyces hyaluronidase, Benzonase® and prostaglandin E1 were from Sigma Aldrich, St. Louis, MO. Thrombin Receptor Activating Peptide (TRAP-6) was from Tocris Bioscience, UK. Anion exchange spin columns were from Thermo Scientific, Waltham, MA. Hyaluronan ELISA-like assay was
from Echelon Bioscience, Salt Lake City, UT. Select-HA™ was from Lifecore Biomedical, LLC, Chaska, MN. Vectasheild Mounting Medium with DAPI was from Vector Labs, Burlingame, CA.

3.3.2. Cell isolation and culture

Mucosal intestinal smooth muscle cells (M-SMCs) were isolated from human surgical colon specimens obtained within 2 hours after resection (kindly provided by the Department of Anatomical Pathology, Cleveland Clinic Foundation, Cleveland, OH). The colonic lamina propria (mucosal layer) was isolated and cut into small pieces. The pieces were added to Hanks’ BSS containing 0.15% dithiothreitol (w/v) and stirred for 30 minutes, then washed three times in Hanks’ BSS containing 1 mmol/L EDTA for a total time of 3 hours, and finally washed in Hanks’ BSS alone for 2 hours. The pieces of tissue were then minced into very small pieces, and incubated for 18 hours in Hanks’ BSS containing collagenase and DNase (0.1 mg/ml each), penicillin (250 U/ml), streptomycin (250 μg/ml), and fungizone (0.625 μg/ml). After the enzymatic digestion, the released cells were filtered from the undigested debris using a 100 µm filter. Cells were then cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; fungizone, 0.25 μg/ml), and incubated at 37°C in a 5% CO₂ humidified environment. Cultures were washed extensively after 48 h to remove non-adherent cells and debris. Confluent cell cultures were split at a 1:3 ratio. Cells were used in their first two passages. Cells at 80% confluency were treated with growth media containing 50 μg/ml poly:I:C for 20 hours.

3.3.3. Platelet Isolation
Blood was collected by sterile venipuncture (without use of a tourniquet) directly into tubes containing Acid-Citrate-Dextrose (ACD) from consented healthy donors as approved by the Cleveland Clinic Institutional Review Board. The citrated blood was centrifuged for 20 min at 200g without breaking. The Platelet-Rich Plasma (PRP) layer was then collected, prostaglandin E1 was added to a final concentration of 35 ng/ml, and the PRP centrifuged for 20 min at 600g. The resulting platelet pellet was then washed twice with HBSS containing 35 ng/mL PGE1.

3.3.4. HA Agarose Gel Electrophoreses

Isolated and washed human platelets were resuspended in RPMI media + 2% FBS (300X10^6/ml) and incubated with or without each of the following: Poly:I:C treated SMCs, HA released from poly:I:C treated cells in solution and purified HA (1000 kDa) in solution for 1 hour at 37ºC. Cell layer was collected by digesting with trypsin. After this step all samples were treated the same for HA isolation. Samples were treated with 0.5 µg/ml proteinase K for 18 hours at 60ºC. Samples were then treated with DNase and RNase and concentrated using a speed vac. Samples were then dialysed against water and were subjected to anion exchange spin columns. Samples were made into 0.1 M NaCl then added to the spin columns. Columns were washed with 0.1 and 0.4 M NaCl. HA was eluted with 0.8 M NaCl. Samples were dialyzed against water and concentrated and applied to 0.5% agarose gel. Electrophorsis was carried at 60V for 4 hours. Gels were stained with 0.005% StainsAll in 50% ethanol and destained with 10% ethanol and light exposure [86].

3.3.5. Immunohistochemical staining of platelets
Freshly isolated platelets were fixed in 3.5% paraformaldehyde for 30 min at RT. Fixed platelets were spun onto a poly-l-lysine coated 8-well chamber slide at 250g for 10 min. Permeabilization of platelets was done by adding cold methanol for 5 min. Fixed Specimens were incubated with blocking buffer (2% FBS in HBSS) for 1 h. Specimens were incubated with a rabbit polyclonal antibody against KIAA1199 (ProteinTech, Chicago, IL) overnight. After washing, specimens were incubated with the 568 Alexa Fluor® anti-rabbit secondary antibody for 45 min at RT. Specimens were washed again and Vectashield Mounting Medium with DAPI was added. Images were obtained using Leica upright microscope DM5500 B (Leica, Germany).

3.3.6 Flow Cytometry

Freshly isolated resting platelets were fixed in 1% paraformaldehyde for 2 h at RT. For permeabлизation and intracellular staining, fixed platelets were further incubated with 0.5% saponin. Platelets were washed then incubated for 1 h at RT in flow cytometry buffer (0.1% BSA, 0.05% sodium azide in PBS) with anti-KIAA1199 (ProteinTech) (1:100) and anti-CD16/CD32 (to block the Fc receptors, 1:100). Platelets were washed then incubated with Alexa Fluor® 568 Goat Anti-Rabbit IgG for 30 min at RT. 30,000 events/sample were acquired on a BD Bioscience LSR II (BD Biosciences) flow cytometer. Data was analyzed using FlowJo (Tree Star, Ashaland, OR).

3.3.7. Platelet microparticles and releasate isolation

Platelet microparticles and releasates were isolated as previously described [84]. Briefly, freshly isolated platelets were incubated without or with 15 μM TRAP for 10
minutes at 37°C and centrifuged at 750g for 20 minutes (platelet fraction). The supernatant platelet releasates were placed on ice and centrifuged at 10,000g for 30 minutes at 4°C to obtain the microparticles fraction. Platelet fractions (750g pellet), microparticle fractions (10^4 pellet), and platelet releasates (10^4 g supernatant) were analyzed by western blotting for the presence of KIAA1199.

3.4. RESULTS AND DISCUSSION

3.4.1. Platelets digest HA released from poly:I:C-stimulated M-SMCs, but not purified HA, in solution.

We previously demonstrated that platelets can digest HA from surfaces of activated endothelium, which produces fragmented HA capable of promoting cellular responses [77]. We also showed that platelets cleave HA from the surfaces of activated mucosal intestinal smooth muscles cells (M-SMCs) [89]. Importantly, we demonstrated that platelet-degradation of HA on the surfaces of activated cells occurs through the activity of HYAL2 [89]. HYAL2 is reported to be an acid active enzyme [23]. However, we showed that degradation of purified HA by platelets and platelet lysates only occurs under acidic conditions, with a peak activity at pH of 3.5. We therefore hypothesized that activated M-SMC-associated HA has a unique property that allows it to be degraded by platelets. To test this hypothesis, we stimulated M-SMCs with poly:I:C for 18 hours to induce pathological HA cable formation. Then, we replaced media and allowed M-SMCs to release their HA for 4 hours. Following incubation, we collected the released HA and incubated it with platelets for 4 hours at 37°C. As controls, we incubated platelets with poly:I:C stimulated M-SMCs and with purified HA in solution. Isolated HA was
analyzed by agarose gel electrophoresis. As expected, under neutral pH, platelets degraded HA from surfaces of activated M-SMCs and did not degrade purified HA in solution. Importantly, however, platelets degraded M-SMC-HA in solution under neutral pH (figure 3.1). Our data suggest a role for either HA binding proteins, HA tertiary structure, or both in facilitating the fragmentation reaction. This implies that HA becomes a better substrate for platelet degradation under inflammatory conditions because the main difference between purified HA and pathological HA matrices is that pathological HA, which is also highly leukocyte adhesive, is associated with HA-binding proteins such as IαI heavy chains and versican.
Figure 3.1: Platelets degrade cell-released HA in solution. Agarose gel electrophoretic analysis of HA before (lanes 1,3,&5) and after (lanes 2,4,&6) incubation with platelets. Whereas commercial purified HMWHA was not degraded by platelets in solution (lanes 5&6), HA released from poly:I:C-treated cells was degraded by platelets at 37°C in a cell-free environment (lanes 3&4). As a positive control, platelets were incubated with M-SMCs and were able to degrade HA from the surfaces of these cells (lanes 1&2). All incubations were carries at pH 7.4.
3.4.2. Platelet activation results in increased platelet-HA binding.

We showed previously that platelet HYAL2 is stored inside α-granules in resting platelets. However, upon activation by a thrombin receptor stimulator, platelet HYAL2 becomes surface expressed, as determined by flow cytometry and immunoelectron and immunofluorescence microscopy. Importantly, platelet activation results in a significant increase in platelet HAase activity, whether the substrate is cell-surface HA or purified HA. We also observed when we blocked platelet HYAL2, platelets retained the ability to bind HA cables, without cleaving them [89]. Collectively, we hypothesized that not only does platelet activation have a role in platelet HAase activity, but also it has a role in platelet-HA binding. To test this hypothesis, we incubated fluorescein isothiocyanate (FITC)-labeled HA with resting and activated platelets. We then measured HA binding to platelets by measuring platelet surface-fluorescence intensity by flow cytometry. We found that resting platelets demonstrated low levels of binding to HA. However, the binding ability of activated platelets to HA was significantly higher (figure 3.2). Upon vascular injury, activated platelets adhere to the proteins of the exposed connective tissues. HA is a major component of the extravascular connective tissues. Therefore, our data provide evidence that HA also represents a substrate for activated platelet binding, probably through a HA binding protein that becomes expressed on the surfaces of platelets upon activation.
Figure 3.2: Activated platelets have higher affinity to HA than resting platelets. The figure shows flow cytometry analysis of mean fluorescence intensity on platelet surfaces upon the incubation of platelets with FITC-labeled HA. Although resting platelets showed binding affinity to HA, TRAP-activated platelets demonstrated significantly higher HA-binding ability to HA.
3.4.3. Platelets express the novel HA-binding protein KIAA1199.

Platelets and their megakaryotic precursors express the enzyme HYAL2 [77]. Importantly, we showed that platelets use their HYAL2, after they become activated, to cleave HA from the surfaces of mucosal intestinal endothelial and smooth muscles cells [77,89]. Additionally, we showed that platelets can bind HA and that this binding activity increases significantly when platelets are activated with a thrombin receptor stimulator. However, how platelets bind HA is still unclear, and little evidence exists that the classic HA receptor CD44 is expressed on human platelets. Therefore, we wanted to investigate the possibility that platelets express a different HA-binding protein. We tested whether platelets express the novel protein KIAA1199, which has just been recently identified as a HA-binding protein that contributes to HA degradation [30]. Using an immunoblotting assay, we found that platelets express KIAA1199, which appeared as a band at the expected molecular weight of 150 kDa. We used two different antibodies that recognize two different amino acid sequences (aa 31-386 and aa 880-890) to eliminate the possibility of non-specific binding (figure 3.3A). Interestingly, both antibodies recognized other bands with smaller sizes, which might represent cleaved forms of KIAA1199. We also confirmed that platelets express KIAA1199 using immunofluorescence microscopy (figure 3.3B). The presence of KIAA1199 in platelets may assist in defining a novel mechanism by which platelets bind HA.
Figure 3.3: Platelets express KIAA1199. (A) Immunoblot analysis of platelet lysates for the presence of KIAA1199. Platelets express the 150 kDa-sized KIAA1199. The presence of KIAA1199 was confirmed using two different antibodies: antibody 1 (Ab1) recognizes amino acids 880-890, whereas antibody 2 (Ab2) recognizes amino acids 31-386. The smaller bands may be indicative of cleaved forms of KIAA1199.
Figure 3.3 (cont.): Platelets express KIAA1199. (B) Immunofluorescence analysis of KIAA1199 (red) expression in platelets. Platelets were fixed and centrifuged onto poly-l-lysine-coated slides. After blocking, platelets were incubated with or without (2° ab only control) an antibody against KIAA1199, washed, and incubated with 568 Alexa secondary antibody. KIAA1199 expression in platelets appears to be granular, suggesting its storage inside platelet granules.
3.4.4. Platelets express KIAA1199 intracellularly and release it upon activation.

Platelet HYAL2 degrades HA from the surfaces of activated intestinal mucosal cells [89]; however, the mechanism by which platelets bind HA is still unclear. We provided evidence that platelet-HA binding increases significantly when platelets become activated. We also showed by immunoblotting and immunofluorescence microscopy that KIAA1199, which is a novel HA-binding protein and a contributor to HA degradation, is expressed by platelets. KIAA1199 has been reported to be localized inside endoplasmic reticulum in cancer cells [91]. We wanted to investigate whether platelet KIAA1199 is expressed intracellularly or extracellularly. Furthermore, we wanted to investigate how platelet activation affects platelet KIAA1199 localization. Activated platelets release numerous adhesive and clotting proteins from their granular stores to the surrounding environment. These molecules can be soluble, associated with the platelet surface, or packaged in microparticles [59]. When we analyzed platelet surface expression of KIAA1199 by flow cytometry, there was little to no KIAA1199 expression detected. However, when we permeablized platelets, we were able to detect KIAA1199 by flow cytometry (figure 3.4A), which suggests that KIAA1199 is stored intracellularly in resting platelets. To investigate the role of platelet activation in the expression of platelet KIAA1199, we activated platelets for 10 minutes using TRAP and isolated their microparticles and soluble releasate. We found that KIAA1199 is completely released upon activation, but does not become part of microparticles (figure 3.4B). Our data suggest that KIAA1199 is a soluble protein that platelets release immediately after activation. However, the role of platelet KIAA1199, and specifically its role in HA binding and depolymerization, is still unclear. Defining the role of platelet-released
KIAA1199 may reveal a novel mechanism by which platelets interact with the extracellular matrix of exposed tissues and a possibly role of platelet in inflammatory diseases, especially IBD.
Figure 3.4: Platelets express KIAA1199 intracellularly. (A) Flow cytometry analysis of surface and intracellular KIAA1199. No KIAA1199 is detected on the surfaces of resting, non-permeabilized platelets. However, significant levels of KIAA1199 staining were obtained in permeabilized platelets, suggestive of intracellular localization of KIAA1199 in resting platelets.
Figure 3.4 (cont.): **KIAA1199 becomes part of platelet releasate upon activation.** (B)

Western blot analysis of KIAA1199. Platelets were activated with TRAP for 10 minutes before they were centrifuged at 750 g to collect platelet pellet. Supernatant was then centrifuged at 10,000 g to collect platelet microparticles. Lanes: (1) non-activated platelet lysate, (2) activated platelet lysate (pellet + supernatant), (3) activated platelets only (750 g pellet), (4) microparticles (10,000 g pellet), (5) platelet releasate (10,000 g supernatant).
CHAPTER IV

DISCUSSION, FUTURE DIRECTIONS, AND CONCLUDING REMARKS

4.1. DISCUSSION

In this study, we showed that platelets degrade HA from the surfaces of activated human intestinal mucosal smooth muscle cells under experimental physiological pH and that this degradation is HYAL2-dependent because blocking HYAL2 abrogated the degradation activity. We also demonstrated that platelet cleavage of purified HA only occurs under acidic pH, which is consistent with previous reports on HYAL2 activity [92]. We found little evidence of HYAL2 on the surfaces of resting platelets, although HYAL2 is known to be a cell surface protein [93]. Instead, HYAL2 was detected exclusively on the surfaces of activated platelets. Importantly, we showed that HYAL2 is packaged within a distinct subpopulation of α-granules in resting platelets, and that upon activation, it gets translocated from α-granules to the platelet surface. While HYAL2 is on platelet surface, it functions to digest HA. We also demonstrated a clinical relevance for platelet HYAL2. We showed evidence that patients with IBD have lower platelet HYAL2 contents than their healthy controls. Additionally, platelets from IBD patients
demonstrated lower hyaluronidase activity than platelets from healthy controls. We also
showed in this report that platelets, under neutral experimental conditions, have the
ability to degrade HA released from activated mucosal intestinal smooth muscle cells, but
not purified HA and that activated platelets have significantly increased binding affinity
to HA in comparison to resting platelets. Finally, we demonstrated that platelets express
the newly discovered HA-binding protein KIAA1199 and release it upon activation.

Our data raise several interesting questions. The observation that platelets degrade
cell surface-HA but not purified HA under physiological pH suggests that platelet-
mediated HA degradation depends upon multiple factors and is likely a regulated process.
HYAL2 is reported to be an acid active enzyme [23]. However, it was demonstrated, in
an embryonic kidney cell line, that HYAL2 becomes active under physiological pH only
when it coexists with CD44 at the plasma membrane [29]. Another group has shown, in a
breast cancer line, that the CD44-dependent HA degradation was achieved due to an
increase in the activity of the sodium-hydrogen exchanger NHE1, causing a local drop in
pH, which creates an acidic microenvironment for HYAL2 [28]. This is an unlikely
scenario in platelets because intact platelets, either resting or activated, did not degrade
purified HA under neutral pH. Additionally, there is no evidence that human platelets
express CD44. In our lab, platelet degradation of HA was not inhibited when we pre-
incubated platelets with anti-CD44 antibodies (data not shown). In our cell co-culture
model, a likely scenario is that HA binding proteins (e.g., versican, pentraxin 3, TSG6,
and the heavy chains of inter-α-trypsin inhibitor) [94,95] may be necessary to facilitate
the interaction between platelets and HA and therefore mediate HA catabolism by
platelets. This is similar to the requirements our group has noted for non-activated leukocyte binding to HA cables [51].

Another possible non-CD44-dependent mechanism by which platelets degrade HA under neutral pH is by the involvement of a platelet HA receptor or binding protein in assisting HYAL2 to degrade HA. A good candidate is KIAA1199, which is a newly identified HA binding protein that specifically contributes to HA degradation. We investigated whether platelets express KIAA1199 and found that platelets have KIAA1199 and store it intracellularly. However, platelets completely release KIAA1199 after they are activated. Additionally, we found that activated platelets have significantly higher binding affinity to HA than non-activated platelets. In some inflammatory diseases, like IBD, HA becomes associated with proteins to form pathological HA structures known as HA cables; in addition, platelets circulate in a highly activated state. For those reasons, our data suggest that HA becomes a better substrate for platelet degradation under inflammatory conditions.

Another interesting point our data raises is that HYAL2 is stored in a distinct subpopulation of α-granules, which lacks three known α-granule markers: P-selectin, vWF, and fibrinogen. Selective packaging of proteins within different subpopulations of α-granules in platelets has been a matter of controversy [96,97]. For example, vascular endothelial growth factor, a pro-angiogenic protein, was shown to be stored in a different α-granule population than endostatin, an anti-angiogenic protein [62]. Conversely, it has been proposed that random distribution of proteins into α-granules occurs [98]. However, the clear localization of HYAL2 within α-granules, observed by electron microscopy and sucrose density gradient analysis, and the complete lack of co-localization between
HYAL2 and P-selectin, a protein thought to be present in all α-granules [62], favor the hypothesis that α-granule proteins can be separated into distinct subpopulations.

Under healthy conditions, platelets do not adhere to the vascular wall. However, upon vascular injury, platelets are exposed to components of extracellular matrix (e.g. collagen and fibronectin), to which they rapidly adhere [99]. We have reported previously that platelets can bind and degrade HA from the surfaces of activated endothelial cells [77]. Here we show that HA in the extracellular matrix of polyI:C treated M-SMCs can also be degraded by platelets. This is important because HA is upregulated during inflammation in many tissues, and smooth muscle cells and myofibroblasts are considered to be the major HA producing cells. High levels of HA deposition have been reported in several inflammatory diseases including asthma, arthritis and hepatitis [44]. Our group, in addition, has reported an upregulation of HA in the intestines of patients with IBD, a chronic condition characterized by tissue erosion and intestinal bleeding [43].

IBD is also associated with platelet dysfunction [75]. Multiple platelet abnormalities have been identified in patients with IBD including reactive thrombocytosis, low mean platelet volume and increased density [71-75]. Importantly, platelets in IBD patients circulate the periphery in an increased activated state and are more susceptible to activation than platelets from healthy subjects [71]. Here, we present yet another abnormality associated with platelets from patients with IBD.

4.2. FUTURE DIRECTIONS

The hypothesis that HA binding proteins that are part of HA cables under inflammatory conditions facilitate the interaction between platelets and HA needs to be
further investigated because not only can it explain how platelets bind HA, but also how other immune cells, like leukocytes, bind HA. A good strategy would be to test whether proteinase K-treated pathological HA structures, which are released into cultured media of poly:I:C-stimulated smooth muscle cells, can be degraded by platelets in solution. Furthermore, these pathological HA structures can be purified and the identity of HA binding proteins can be determined by mass spectrometric analysis. Identifying the specific HA-binding protein that facilitates the interaction between platelets and HA can be crucial to understanding the conditions under which platelets bind and degrade HA.

Also, because we found that HYAL2 is an α-granule protein that does not colocalize with any known classical α-granule protein, we concluded that HYAL2 is stored in a distinct subpopulation if α-granules. Further research, however, is needed to define the contents of the HYAL2-containing α-granules and to determine whether any functional coclustering of proteins within these granules exists. One approach to investigate the contents of HYAL2-containing α-granules would be to isolate these specific granules using a HYAL2 antibody bound to protein-A-coated magnetic beads. After the granules are purified, they can be lysed and their contents can be determined by mass spectrometry. It would be interesting to know whether KIAA1199 is located inside these granules along with other possible HA binding proteins or receptors. Furthermore, the possibility that HYAL2 binds to KIAA1199 needs to be investigated, possibly by performing co-immunoprecipitation experiments.

On the other hand, it might be critical to further investigate the hypothesis that platelet HYAL2 has a role in IBD. IBD is a very complex disease and understanding the role of platelets in its pathogenesis might provide new directions on how to treat the
disease. One method to investigate the role of platelet HYAL2 in IBD is to induce colitis in a HYAL2-knockout mouse and study the pathology and progression of the disease in this mouse model. It also might be important to determine whether transfusing platelets from wild-type mice into the colitis HYAL2-knockout mice results in faster recovery. Finally, because most currently used methods to diagnose IBD are invasive, investigating the possibility of using low platelet HYAL2 and activity as biomarkers for IBD may be important in the diagnosis of this enigmatic disease.

4.3. CONCLUDING REMARKS

Studies on the interaction between platelets and the ECM have been limited to the interaction between platelets and ECM proteins, including collagen and fibrinogen. Here, we present a novel mechanism by which platelets interact with ECM; we show that platelets can interact also with a polysaccharide component of the ECM, which is HA. This interaction may be crucial to the pathogenesis of IBD and possibly other inflammatory diseases. Figure 41 shows how low platelet HYAL2 and HAase activity might have a role in the pathogenesis of IBD. One possibility is that the lack of HYAL2 in platelets might be contributing to intravascular microthrombi formation, a feature widely observed in IBD [100]. This major abnormality in IBD mucosa is characterized by the presence of platelet thrombi cross-linked with fibrin in the mucosal microvasculature. Interestingly, it has been reported that HA can tightly bind fibrinogen [101,102]; in addition, there is a significant increase in HA deposition in tissues of IBD patients [43]. Collectively, HYAL2 might be necessary to degrade HA and therefore prevent thrombus formation.
We previously showed that the accumulating pathological HA in the tissues of IBD patients has the ability to bind and recruit leukocytes [51], which, in turn, can secrete various inflammatory cytokines. Low platelet HYAL2 might allow for increased accumulation of HA, resulting in increased number of leukocytes recruited. Cytokines release by leukocytes may create a chronic condition in IBD by stimulating vascular cells to produce more pathological HA cables, which, in turn, bind more leukocytes creating a cycle of inflammation.

Another mechanism by which HYAL2 might be contributing to IBD is its possible role in wound healing. Ulceration due to severe inflammation is a major feature in IBD and can cause damage to blood vessels. HA sharply accumulates during the inflammation phase of wound healing and impaired clearance of HA may result in enduring inflammation [103]. As a result, HA degradation by platelet HYAL2 might be crucial for proper tissue repair upon injury. Furthermore, HA fragments generated by platelet HYAL2 may have a role in improving wound healing [104]. All of these functions (the prevention of microthrombi formation, the prevention of leukocyte recruitment, and wound healing) could be impaired in IBD patients with low platelet HYAL2.
Figure 41. Low platelet HYAL2 contributes to IBD pathogenesis. Low platelet HYAL2 may contribute to IBD pathogenesis by three different ways: 1) Low platelet HYAL2 results in increased HA accumulation. The ability of platelets to bind HA and fibrinogen, in addition to the ability of HA to bind fibrinogen, may result in microthrombi formation, typically observed in tissues of patients with IBD; 2) Low platelet HYAL2 results in increased HA cables that can bind leukocytes, which produce cytokines that create an inflammation cycle; 3) Low platelet HYAL2 results in increased HA accumulation and decreased HA fragments. Both of which may result in impaired wound healing.
BIBLIOGRAPHY


107


53. de La Motte CA, Hascall VC, Calabro A, Yen-Lieberman B, Strong SA. Mononuclear leukocytes preferentially bind via CD44 to hyaluronan on human


