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"Never express yourself more clearly than you are able to think" – Niels Bohr

Perheelleni ja muille tärkeille ihmisille

TIIVISTELMÄ

Integriinit ovat solunpinnan tarttumis- ja signalointireseptoreja, joiden avulla solut kiinnittyvät ja välittävät tietoa ympäristöstään. Integriineillä on osoitettu olevan keskeinen rooli solujen tarttumisen ja liikkumisen lisäksi mm. ohjelmoidussa solukuolemassa eli apoptoosissa, solujakaantumisessa sekä solujen erilaistumisessa kantasoluista eri kudostyypeiksi. Monet uuden sukupolven biologiset lääkkeet, joita käytetään esimerkiksi MS-taudin ja verihyytymien hoidossa, kohdistuvat integriinien toiminnan estämiseen. Näin ollen integriinien ja niiden ligandien välisten vuorovaikutusten tutkiminen erilaisissa fysiologisissa ja patologisissa tilanteissa on tärkeää perustutkimuksellisen mielenkiintoisuuden lisäksi myös lääkekehityksen kannalta.

Tässä työssä integriinien toimintaa on tutkittu tavanomaisesta integriini-ligandi-vuorovaikutuksesta poikkeavissa tilanteista. Tälläisia tilanteita ovat 1) echovirus 1:n vuorovaikutus integriini α2β1 kanssa, 2) integriini α2β1:n sitoutuminen kollageeniin virtauksen alaisena, 3) integriinien ligandiin tarttumisen estyminen angiogeneesi-inhibiittori HRG-proteiinin (eng. histidine-rich glycoprotein) vaikutuksesta sekä 4) integriini-ligandien aminohappojen muokkautuminen translaation jälkeisesti sitrulliniiksi niveltulehduksissa. Väitöskirjatyön tuloksena havaittiin, että kussakin tilanteessa integriinien sitoutuminen ligandiinsa poikkesi tavanomaisesta käsityksestä: 1) echovirus 1 tarttuu vain inaktiiviseen α2β1-integriinin konformaatioon ja yllätyksellisesti 2) α2β1 sitoutuu virtauksen alaisena kollageeniin käyttämällä inaktivoitua konformaatiota. Myöskään integriinin pre-aktivaatiolla ei näyttänyt olevan merkitystä integriini α2β1:n sitoutumiseen kollageenin virtauksellisissa olosuhteissa. 3) HRG:n toimintamekanismin selvitys osoitti, että integriini α2β1 sitoutuu matalalla affiniteetilla HRG-proteiiniin estäen α2β1:n sitoutumisen kollageeniin, mikä osoittaa, että matalan affiniteetin integriini-ligandi-vuorovaikutuksella voi olla biologisesti tärkeitä tehtäviä. Lisäksi havaittiin, että 4) kollageenin sitrullinaatiolla ei ole vaikutusta α1β1 ja α2β1 integriinien sitoumiseen, mutta α10β1 ja α11β1 eivät pysty sitoutumaan sitrullinoituun kollageeniin. Toisaalta esim. fibronektiinin isoDGR- sekä pro-TGF-β:n RGD-motiivin sitrullinoituminen estää integriinien sitoutumisen kokonaan. Sitrullinaatio vaikuttaa olevan yleinen niveltulehdukseen liittyvä prosessi, ja monia integriinien sitoutumiskohtia ligandeissa sitrullinoidaan aktivoitujen neutrofiilien kerääntyessä tulehdukseen *in vivo*.

Väitöskirjatyö osoittaa, että epätavalliset integriini-ligandi-vuorovaikutusmekanismit, kuten ligandien translaation jälkeiset modifikaatiot, matala-affiiniset integriini-ligandivuorovaikutukset sekä inaktiiviseen konformaatioon sitoutuminen, näyttäisivät olevan keskeisessä osassa sairausprosesseissa. Tällaisten integriini-ligandi-interaktioiden tuntemus on tärkeää, jotta voidaan ymmärtää biologisten prosessien toimintaa syvällisemmällä tasolla. Tutkimus auttaa mahdollisesti myös kehitettäessä integriineihin liittyviä terapioita sairauksien hoitoon.

ABSTRACT

Integrins are cell surface adhesion and signaling receptors. Cells use integrins to attach to the extracellular matrix and to other cells, as well as for sensing their environment. In addition to adhesion and migration, integrins have been shown to be important for many biological processes including apoptosis, cell proliferation, and differentiation into specific tissues. Many important next generation biological drugs inhibit integrin functions. Thus, research into interactions between integrins and their ligands under different physiological and pathological conditions is not only of academic interest, but is also important for the field of drug discovery.

In this Ph.D. project, the functions of integrin-ligand interactions were studied under different physiologically interesting conditions including 1) human echovirus 1 binding to integrin α2β1, 2) integrin α2β1 binding to collagen under flow conditions, 3) integrin α2β1 binding to a ligand in the presence of the angiogenesis inhibitor histidine rich glycoprotein (HRG) and 4) integrin binding to posttranslationally citrullinated ligands.

As a result of the project, we could show that for each condition the integrin-ligand interaction is somewhat unconventional. 1) Echovirus 1 binds only to non-activated conformations of integrin α2β1. 2) Surprisingly, the non-activated conformation is also the primary conformation of integrin α2β1 when it binds to collagen under flow conditions, like when platelets adhere to subendothelial collagen in vascular injuries. In addition, the pre-activation of integrin α2β1 does not increase adhesion under flow. 3) HRG binds to integrin α2β1 through a low-affinity interaction that inhibits integrin binding to collagen. This shows that low affinity interactions could be biologically relevant and possibly regulate angiogenesis. 4) The citrullination of collagen, a posttranslational modification reported to occur in rheumatoid arthritis, specifically inhibits the binding of integrin α10β1 and α11β1, but does not affect the binding of α1β1 ja α2β1. On the other hand, the citrullination of isoDGR in fibronectin and RGD in pro-TGF- β:n inhibit integrin binding completely. Citrullination seems to be an inflammation related process and integrin ligands become citrullinated frequently *in vivo*.

This Ph.D. thesis suggests that unconventional interaction mechanisms between integrins and their ligands, such as posttranslational modifications, low affinity interactions, and non-activated integrin conformations, can have an important role in pathological processes. The study of these kinds of integrin-ligand interactions is important for understanding biological phenomena more deeply. The research might also be beneficial for the development of integrin based therapies for treating diseases.

CONTENTS

ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
BSA	Bovine serum albumin
CII	Collagen II
CHO	Chinese hamster ovary
CIA	Collagen induced arthritis
Cit/cit	Citrulline
Col	Collagen
CPS	Counts per second
DDR	Discoidin domain receptor
DMARD	Disease-modifying antirheumatic drugs
ECM	Extracellular matrix
EV1	Echovirus 1
FAK	Focal adhesion kinase
GPVI	Glycoprotein VI
GSK3β	Glycogen synthase kinase-3β
HRG	Histidine-rich glycoprotein
Htase	Heparitinase
ILK	Integrin-linked kinase
Kd	Dissociation constant
LAIR-1	Leukocyte-associated immunoglobulin-like receptor I
LAP	Latency associated peptide
LLC	Large latent complex
LTBP	Latent TGF-β binding protein
MAC	Membrane attacking complex
MBP	Myelin basic protein
MLEC-PAI-1/Lu	TGF-β reporter cell line
MS	Multiple sclerosis
NET	Neutrophil extracellular trap
NETosis	Formation and release of neutrophil extracellular traps
O	Hydroxyproline
PAD	Peptidyl arginine deiminase
PDB	Protein Data Bank
PIMT	Protein isoaspartyl methyltransferase
PPAD	*Porphymonas gingivalis* peptidyl arginine deiminase
PSM	Peptde spectrum match
PTPN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
RA	Rheumatoid arthritis
RGD	Arginine-glycine-aspartate
SNP	Single nucleotide polymorphism
SLC	Small latent complex
TGF-β	Transforming growth factor beta
TGF-RI/II	TGF-β receptor I/II
VLA	Very late antigen
WBC	White blood count

LIST OF ORIGINAL STUDIES

This thesis is based on the following original publications and manuscripts, referred to in the text by their Roman numerals:

I. Jokinen J, White DJ, Salmela M, Huhtala M, Käpylä J, **Sipilä K**, Puranen JS, Nissinen L, Kankaanpää P, Marjomäki V, Hyypiä T, Johnson MS, Heino J (2010) Molecular mechanism of α2β1 integrin interaction with human echovirus 1. EMBO J 29(1):196-208.

II. Nissinen L, Koivunen J, Käpylä J, Salmela M, Nieminen J, Jokinen J, **Sipilä K**, Pihlavisto M, Pentikäinen OT, Marjamäki A, Heino J (2012) Novel α2β1 integrin inhibitors reveal that integrin binding to collagen under shear stress conditions does not require receptor pre-activation. J Biol Chem 287(53):44694-702.

III. Roche F*, **Sipilä K***, Honjo S, Johansson S, Tugues S, Heino J*, Claesson-Welsh L* (2015) Histidine-rich glycoprotein blocks collagen-binding integrins and adhesion of endothelial cells through low-affinity interaction with α2 integrin. Matrix Biol 48:89-99.

* Equal contribution by first and last authors

IV. **Sipilä K**, Haag S, Denessiouk K, Käpylä J, Peters EC, Denesyuk A, Hansen U, Konttinen Y, Johnson MS, Holmdahl R, Heino J (2014) Citrullination of collagen II affects integrin-mediated cell adhesion in a receptor-specific manner. FASEB J 28(8):3758-68.

V. **Sipilä KH**, Ranga V, Rappu P, Torittu A, Pirilä L, Käpylä J, Johnson MS, Larjava H, Heino J (2015) Extracellular citrullination inhibits the function of matrix associated TGF-β. Submitted manuscript.

VI. **Sipilä KH**, Ranga V, Rappu P, Mali M, Pirilä L, Heino I, Käpylä J, Johnson MS, Heino J (2016) Functional arginine residues of extracellular proteins are targets of citrullination *in vivo* in human joint inflammations. Manuscript.

1. INTRODUCTION

1.1. The integrin family of cell adhesion receptors

Integrins are cell surface adhesion receptors and their main function is to mediate adhesion between cells and the extracellular matrix (Hynes 1992), but they also influence cell–cell contacts (Salmi and Jalkanen 1991). In addition, integrins act as two-way signaling receptors transmitting signals from the outside of the cell to the inside and vice versa (Shattil et al. 2010). In terms of cellular functions, integrins are important during development (Fässler and Mayer 1995) and in different physiological and pathological states in adults, as they participate in apoptosis, proliferation, migration, angiogenesis and the activation of growth factors (Barczyk et al. 2010, Ivaska and Heino 2011). Because of their major role in many diseases, integrins are potential targets for drug development (Goodman and Picard 2002).

1.1.1. The discovery of integrins

The importance of the extracellular matrix in regulating cell behavior was proposed as early as in 1955 (Grobstein, 1955). These studies in developmental biology clearly demonstrated that the extracellular matrix is a driving component of "embryonic induction" (Hay, 1977). Thus, it seemed that cell adhesion is more than simply the passive attachment of cells. The first clear indication that the extracellular matrix component can directly guide gene expression came from a study where collagen was shown to affect the differentiation of myoblasts into myotubes (Hauschka and Konigsberg 1966).

The key steps leading to a further understanding of cell adhesion were the discovery of fibronectin, an important ligand for integrins, by several laboratories in the 1970s (Hynes 1973, Gahmberg and Hakomori 1973, Ruoslahti et al. 1973, Ruoslahti and Vaheri 1974) and the characterization of the RGD sequence in fibronectin as a central cell adhesion motif (Pierschbacher and Ruoslahti 1984). By the beginning of 1980 it had become clear that the effect of the extracellular matrix had to be mediated by cell surface receptors (Bissell et al. 1982). During the next two years to follow, the first integrins were found using different experimental approaches. In 1985 an integrin was purified with RGD-peptide based affinity chromatography and was shown to be a 140 kDa glycoprotein (Pytela et al. 1985). At the same time, another approach was used to generate monoclonal antibodies that blocked cell adhesion to fibronectin and laminin (Horwitz et al. 1985). The affinity purification of the antigen revealed that its properties were similar to those of the RGD-purified protein. The first integrin-type cell adhesion receptor was cloned one year later (Tamkun et al. 1986). All of the known integrin family receptors were cloned and characterized during the 1980s and 1990s (Sheppard 2000).

The regulatory role of integrins in cell behavior, such as in differentiation, was shown in the late 1980s (Menko and Boettiger 1987, Adams and Watt 1989) following the characterization of many key intracellular integrin signaling pathways (Lipfert et al. 1992, Chen et al. 1994, Hannigan et al. 1996, Giancotti and Ruoslahti 1999) as well as the identification of integrins' key role in activating transforming growth factor beta (TGF-β) (Munger et al. 1998).

1.1.2. Integrin structure and ligands

Integrin-type adhesion receptors are expressed by every multicellular organism and thus they appeared early in metazoan evolution (Johnson et al. 2009). They are heterodimeric proteins consisting of one α and one β subunit (Heino et al. 1989). The numbers of different α and β subunits vary among organisms. For example, fruit flies have five α subunits and two β subunits, whereas humans have 18 α subunits and 8 β subunits. Twenty-four different combinations of α and β subunit form the heterodimeric integrin receptor (Table 1, Bökel and Brown 2002 and Johnson et al. 2009).

The general domain structure of an integrin is presented in Figure 1. Both integrin subunits have a big extracellular domain, a single transmembrane domain, and a small cytoplasmic tail (Adair and Yeager 2002, Luo and Springer 2006, Hynes 2002). Integrins bind to their ligand via their headpiece that is formed by an interaction between the α and β subunits. The αI domain containing integrins (Table 1.) bind to their ligands with an inserted von Willebrand factor type A domain. In the case of RGD integrins, ligand binding occurs via the homologous I domain located in the β subunit and the β-propeller of the α subunit. The binding of an integrin to its ligand is Mg^{2+} or Mn^{2+} dependent. Based on X-ray crystallography structures, an integrin can exist in at least three different conformations: a bent conformation with a closed headpiece, an extended conformation with a closed headpiece, and an extended conformation with an open headpiece. The extended conformation with an open headpiece is needed for full binding affinity and it has been thought that in order to bind large ligands integrins need to be fully activated (Luo and Springer 2006, Hynes 2002). A cell's ability to bind the extracellular matrix is also regulated by integrin clustering and recycling (Connors et al. 2007, Pellinen et al. 2008)

The regulation of integrin affinity based on conformation is coupled to bidirectional integrin signaling. This means that integrins become conformationally activated by an intracellular signal but that on the other hand, ligand-bound integrins can transmit the signal into the cell (Ivaska and Heino 2011). Interestingly, integrins can also sense mechanical forces related to ligang binding (Trappmann et al. 2012, Doyle and Yamada 2015). Signaling and conformational regulation are mediated by cytoplasmic protein complexes that bind to the tail of integrins. Some of the components that bind the tail can activate the integrins (talin, kindlins) or inactivate them (sharpin) as

well as regulate their endocytosis (Shattil et al. 2010, Rantala et al. 2011, Pellinen et al. 2008). Focal adhesion kinase (FAK) and a pseudokinase called integrin-linked kinase (ILK) are the most important downstream signal-transducing proteins that bind to the cytoplasmic domains of integrin, but also many other kinases, phosphatases, GTPases and adaptor proteins exist. The Ras-MAPK (mitogen-activated protein kinase) and phosphoinositide 3-kinase (PI3K)-Akt signaling pathways are typical examples of downstream pathways for integrins (Ivaska and Heino 2011). Integrin signaling is not restricted to the plasma membrane but can also take place in endosomes (Alanko et al. 2015).

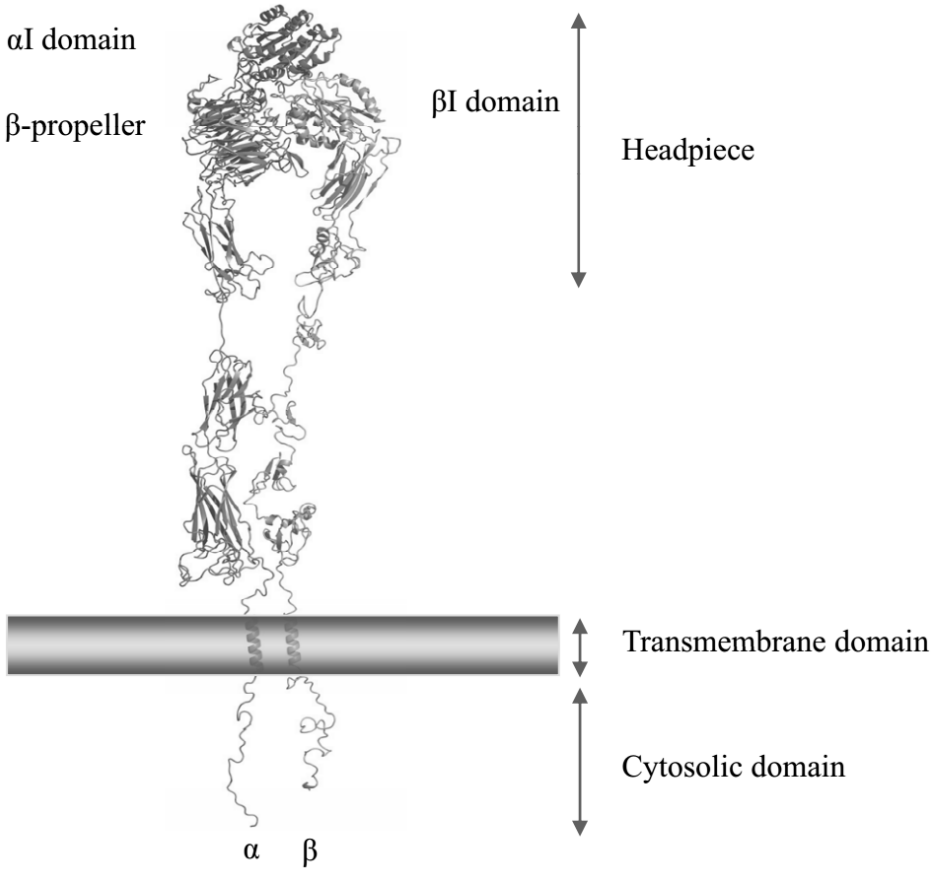


Figure 1. General domain structure of an αI domain containing integrin modelled by Mikko Huhtala (figure modified from Jokinen 2010).

Traditionally, integrins have been divided into four different classes based on how they bind to their ligands (Table 1). The groups are: RGD-binding integrins, LDV-binding integrins, non-I-domain-containing laminin binding integrins, and αI-domain containing integrins (Humphries et al. 2006, Johnson et al. 2009). Integrins within a group share a similar kind of ligand binding mechanism at least partly, but this

nomenclature does not reflect the evolutionary origin of the receptors. The ligands of RGD-binding integrins include extracellular matrix proteins and soluble ligands that have an RGD motif, like fibronectin, vitronectin, and fibrinogen. The ligands of LDV-binding integrins are, for example, immunoglobulin superfamily adhesion proteins VCAM1 (vascular cell adhesion molecule 1) and MadCAM (mucosal vascular addressin cell adhesion molecule 1) but also fibronectin and tenascin C. Despite the fact that β2 integrins have an αI domain that is not found in other LVD-group integrins, the structure of the binding site in their ligands resemble those in the ligads of other LVD-group integrins. The major ligands for the group of αI-domain containing integrins are collagens (Humphries et al. 2006).

Another way of dividing integins into different subgroups is based on the evolutionary relationship between the α-subunits of human and *Drosophila melanogaster* integrins (PSs) (Table 1): the PS1 group, the PS2, group, the α4/α9 group, and the αI domain containing group. *D. melanogaster* has also PS3 integrins but they do not have counterparts in human. On the other hand, α4 and α9 integrins form their own subgroup as do αI domain containing integrins. The integrin β subunits can be divided into to two different groups based on evolutionary similarities: vertebrate A (β1, β2, β7) and vertebrate B (β3, β4, β5, β6, β8) (Johnson et al. 2009).

1.1.2.1. Viruses

As cell surface receptors that undergo extensive endocytosis, integrins are attractive targets for viruses as points of entry into cells. Some clinically important adenoviruses, like foot-and-mouth disease, have an RGD on their surface (Fox et al. 1989; Mathias et al, 1994). In addition to RGD-mediated binding, a virus can interact with an integrin through various different mechanisms. For example, the human echoviruses 1 (EV1) and 8 can bind to integrin α2β1 (Bergelson et al. 1992 and Kawaguchi et al. 1994).

1.1.2.2. Collagens

Collagens are the major group of extracellular matrix proteins and also integrin ligands (Heino 2007). They are proteins that have a region containing G-X-Y repeats in the primary structure of the polypeptide, which enables the formation of a triple helical structure with other collagen polypeptides (Myllyharju and Kivirikko 2004). The collagen helix was among the first protein structures ever to be determined (Rich and Crick 1955). Collagens are the most abundant proteins in humans. They are the major structural component of the connective tissue and they also anchor cells to tissues (Heino 2007). There are 28 different collagen subtypes (collagen I-XXVIII) encoded by 43 different genes so the most collagens consist of more than one polypeptide chain (Myllyharju and Kivirikko 2004, Veit et al. 2006). The nomenclature of collagens is somehow artificial, because proteins other than collagen, like complement C1q and ectodysplasin, share structural similarities with collagens (Heino 2007).

Table 1. Integrin classification (Humphries et al. 2006, Johnson et al. 2009, Wipff and Hinz 2008, Reed et al. 2015)

	Receptor for fibronectin	Receptor for collagen	Activates TGF-β	RGD binding integrin	LDV binding integrin	I-domain containing collagen receptor (GFOGER-type recognition)	Non-I-domain containing laminin binding integrin
PS1 group of integrin of α subunit							
α3β1							X
α6β1							X
α6β4							X
α7β1							X
PS2 group integrin of α subunit							
α5β1	X			X			
α8β1	X			X			
αVβ1	X		X	X			
αVβ3	X		X	X			
αVβ5			X	X			
αVβ6	X		X	X			
αVβ8			X	X			
αIIbβ3	X			X			
α4/α9 group of α subunit							
α4β1	X				X		
α4β7	X				X		
α9β1	X				X		
αI domain containing group of α subunits							
α1β1		X				X	
α2β1		X				X	
α10β1		X				X	
α11β1		X				X	
αDβ2					X		
αMβ2					X		
αLβ2					X		
αXβ2					X		
αEβ7					X		

Collagens are the targets of intensive posttranslational modifications by intracellular prolyl 3 and prolyl 4 –hydroxylases (Tryggvason et al. 1976) as well as lysyl hydroxylases (Krane et al. 1972) after protein synthesis. Besides these modifications, collagens are cross-linked covalently by lysyl oxidation after secretion and propeptide cleavage (Siegel et al. 1976). The structure of collagen is critically dependent on hydroxylation. Defective prolyl 4-hydroxylation causes abnormalities in collagen stability and synthesis. Scurvy is a disease where collagen hydroxylation is prevented by the lack of vitamin C (Englard and Seifter 1986). Vitamin C, is needed to restore the oxidized heme Fe^{2+} of the prolyl 4-hydroxylase back to its active reduced form (Du et al. 2012). Wide connective tissue damage is typical for/characteristic of scurvy (Grosso et al. 2013). Excessive prolyl hydroxylation increases the melting temperature of the collagen triple helix allowing the formation of the helix at higher temperatures. However, the melting temperature of collagen is some degrees below the physiological temperature (at least in the case of collagen I), which means that the structure of collagen is more of a random coil than a highly ordered triple helix at tissue temperature (Leikina et al. 2002). The collagen chaperone HSP47 helps collagen fold during synthesis, and the unfolding velocity of the collagen triple helix is low enough to allow the formation of collagen fibrils and cross-links (Leikina et al. 2002, Nagata 2003).

Collagens can be divided into eight different groups based on their structure and function. The groups are fibril forming collagens (I, II, III, V, XI, XXIV and XXVII), FACIT collagens (IX, XII, XIV, XVI, XIX, XX, XXI, XXII and XXVI) that are located on the surface of fibrils, collagens that form hexagonal networks (VIII and X), basement membrane collagen (IV), collagens that form beaded-filaments (VI, XXVII), collagens that form anchoring fibrils (VII), transmembrane collagens (XIII, XVII, XXIII and XXV), and multiplexin (XV and XVIII) (Myllyharju and Kivirikko 2004, Veit et al. 2006). The physiological supramolecular structures of collagens usually consist of more than one different collagen (Bruckner 2010) and the atomic structures of these complexes are not very well known (Perumal et al. 2008).

1.1.2.3. Integrin-type collagen receptors

Cell-collagen interactions can be mediated by glycoproteins, such as fibronectin and laminins, but some cellular receptors also bind directly to collagen (Elices and Hemler 1989). The known transmembrane collagen receptors are integrins α1β1, α2β1, α10β1, and α11β1, discoidin domain receptors 1 and 2 (DDRs), glycoprotein VI (GPVI) as well as leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) (Leitinger 2011).

Primarily, integrin α1β1 is expressed in mesenchymal cells, α2β1 in epithelial cells and platelets, α10β1 in chondrocytes, and α11β1 in fibroblasts (Barczyk et al. 2010). The receptors are also expressed in other cell types and, for example, α1β1 and α2β1 were originally found in activated leukocytes and named very late antigen 1 and 2

(VLA-1 and VLA2) (Hemler et al. 1985). Based on the cell biological and early knockout animal studies, the role of collagen receptors for an organism's physiology was considered important (Shepard 2000), but the confirmed phenotypes of the collagen receptor integrin knockout mice appeared to be mild (Barczyk et al. 2010). For example, α1β1 knockouts have no obvious phenotype apart from defective feedback regulation of collagen synthesis in the dermis (Gardner et al. 1999) and reduced callus size in a bone fracture model (Ekholm et al. 2002). The α2β1 knock-out leads to mild problems in thrombosis and to the branching of mammary glands (Chen et al. 2002) as well as reduced angiogenesis (Zhang et al. 2008) and a reduced response by cells of the innate immunity (Edelson et al. 2006). α10β1 knockouts have defects in the chondrocytes of the growth plate (Bengtsson et al. 2005). The α11β1 null mouse has defective incisors (Popova et al. 2007) and problems with wound healing (Schulz et al. 2015). Interestingly, a nonsense mutation in integrin α10 in Norwegian Elkhound and Karelian Bear dogs leads to a complete lack of integrin α10 in cartilage and the animals have relatively severe chondrodysplasia compared to α10β1 knockout mice (Kyöstilä et al. 2013), which may be due to the differences in body mass. Thus, the results obtained with mouse models do not directly reflect the situation in heavier animals.

A technique, that uses overlapping triple-helical collagen mimetic peptides, enabled the more elaborate characterization of the binding motifs of collagen receptors (Knight 1998). At present, the following triple helical integrin binding motifs in collagen are known: GROGER (O=hydroxyproline, collagen I and III; Kim et. al 2005), GLOGER (collagen I and II; Raynal et al. 2006), GFOGER (collagen I and II; Knight et al. 1998), GMOGER (collagen I, II, and III;), GLKGEN (collagen III; Raynal et al. 2006), GLOGEN (collagen III; Raynal et al. 2006), GAOGER (collagen III; Kim et al. 2005), GVOGEA (collagen II; Hamaia et al. 2012), GLSGER (collagen III; Munnix et al. 2008), GLOQGER and GFKGER (collagen XXII; Zwolanek et al. 2014). The crystal structure of the α2 I domain-GFOGER complex reveals that the ligand is bound via the metal ion (the glutamate of the ligand binding to the metal) that is coordinated to the MIDAS site of the α I domain (Emsley et al. 2000). All of the collagen receptor integrins recognize the GFOGER-type binding motif although GVOGEA seems to bind only to the α1I domain (Hamaia et al. 2012). It has been shown that integrin α2β1 binds with high affinity to fibril-forming collagens, whereas α1β1 prefers to bind to collagen IV (Tulla et al. 2001). α2β1 has also been shown to bind to collagen fibrils (Jokinen et al. 2004). The ligand recognition mechanisms of α11β1 and α10β1 seem to be similar to those of α2β1 and α1β1, respectively. The need of hydroxyproline for binding was thought to differ between α2 and α1: α1β1 showed reduced binding to collagen without prolyl hydroxylation but α2β1 bound equally well whether it was prolyl hydroxylated or not (Perret et al. 2003). Still, it has recently been shown that both integrin binds to a non-hydroxylated GPPGER sequence (Seo et al. 2010). Thus, collagen receptor integrins have distinct ligand recognition preferences, and the mechanism for this can be based on the recognition of different binding motifs, different collagen subtypes or differencies in the prolyl hydroxylation state.

1.1.2.4. Fibronectin

Fibronectin is a prototypic cell adhesion molecule that mediates the adhesion of cells to the extracellular matrix by integrins. Besides, fibronectin is a large scaffold protein that binds many growth factors and proteins of the extracellular matrix (Hynes 2009). It is built by different modules known as type I, II and III domains (Fig. 21). There are 12 type I domains, 2 type II domains and 15 type III domains in fibronectin. In addition, some splicing variants have EIIIA, EIIIB (structurally type III domains), or a variable region V. Fibronectin can lack the V region partly or completely. Plasma fibronectin has neither EIIIA nor EIIIB, but the splice variants of cellular fibronectin can have different combinations of the extra domains. Due to alternative splicing, the size of fibronectin varies from 230 kDa to 270 kDa (Schwarzbauer and DeSimone 2011).

The different splicing variants of fibronectin vary functionally and are expressed in different tissues and physiological processes. Mice with separate single domain knockouts of EIIA or EIIIB have a normal life cycle, but the double knock-out causes embryonic lethality (Astrof et al. 2007, Schwarzbauer and DeSimone 2011). The functional differences between the splice variants might be mediated by changes in cell adhesion, but also other explanations are possible. For example, plasma fibronectin is more soluble than the cellular form and the EIIIB forms are cleaved more effectively by proteases (Schwarzbauer and DeSimone 2011).

1.1.2.5. Integrin-type fibronectin receptors

There are a couple of different integrin binding sites in fibronectin and the most well-known is the RGD site in the tenth of type III domain (Pierschbacher and Ruoslahti 1984). Integrins that bind the RGD-site are all αV integrins, α5β1, α8β1, and αIIbβ3 (Leiss et al. 2008). The binding of α3β1 to RGD has also been suggested (Brown et al. 2015). In addition to the RGD site, α5β1 and αIIbβ3, but not αV integrins need the so called synergy region, a PHSRN motif in the ninth of type III domain, for binding with full affinity (Aota et al. 1994, Chada et al. 2006, Leiss et al. 2008). It is known that the synergy region does not make direct contact with integrin α5β1, but the exact mechanism remains to be investigated (Takagi et al. 2003). However, mutations in the synergy region lead to problems in integrin mediated processes in cell biological models as well as in mice (Leiss et al. 2008). The binding of cells with different integrins to fibronectin causes different cellular and physiological responses. A quantitative proteomics approach revealed that β1 integrins trigger the peripheral adhesions by the RhoA-Rock-myosin II pathway and generate the required force. In contrast, αV integrins promote large focal adhesions by formin the mDia1 coupled RhoA guanine nucleotide exchange factor GEF-H1-RhoA pathway and enable adaptations to the force (Schillller et al. 2013).

Mice, in which RGD is mutated to RGE, die on embryonic day 10. They resemble α5 knockout mice in many ways but, surprisingly, the mutant fibronectin can still form a

matrix (Takahashi et al. 2007). The reason for this might be the NGR binding site of αV integrin in fibronectin the fifth of type I domain. The NGR can undergo spontaneous deamidation where asparagine residues are converted into isoaspartates (isoD), forming a high affinity binding site for αVβ3 (Curnis et al. 2006). Still, the NGR/isoDGR seems to be cryptic and does not mediate the binding of the N-terminal 70 kDa fibronectin fragment to the fibroblast surface (Xu et al. 2010). Interestingly, with age fibronectin generates more isoDGR and increases its affinity to integrin. Protein L-isoaspartyl methyltransferase (PIMT) restores the deamidated isoD back to N (Curnis et al. 2006). PIMT can be released from cells as a consequence of injury, which makes the enzyme a potential regulator of cell adhesion (Weber and McFadden PN 1997). Integrins α4β1 and α4β7 bind to the variable region of fibronectin, which is located between the fourteenth and fifteenth of type III domains. The binding is mediated by the LDV motif. In addition, the EDA domain of fibronectin has binding sites for α4β1 and α9β1. In this case, the binding motif is the EDGIHEL sequence (Leiss et al. 2008).

1.1.3. Syndecans

Syndecans are cell surface receptors that are involved in cell adhesion events. The syndecan family consist of four different syndecans called syndecan-1,-2,-3, and -4 (Morgan et al. 2007). Unlike integrins, syndecans are proteoglycans that bind to growth factors and molecules of the extracellular matrix mainly via glycosaminoglycan chains (Saunders and Bernfield 1988, Matsuo and Kimura-Yoshida 2014). Glycosaminoglycans are either heparin or chondroitin sulphate sugar chains. They are covalently bound to the extracellular domain of a syndecan by a 3-5 substitution. The core protein of syndecan has a 29 kDa extracellular domain and a short cytoplasmic tail. It is known that proteins with a PDZ-domain and the proto-oncogene tyrosine-protein kinase Fyn (member of the Src family) bind to different regions (C2 and C1, respectively) of the cytoplasmic tail of syndecan. Besides the C1 and C2 regions, the cytoplasmic domains of syndecans consist of a variable region. In the case of syndecan-4, protein kinase Cα binds to the variable region in the cytoplasmic tail (Morgan et al. 2007).

Interestingly, many extracellular proteins have binding sites for both integrins and syndecans, which indicates some kind of synergy between these two receptor groups when the cell attaches to the extracellular matrix (Morgan et al. 2007). In the case of integrin α2β1, an interaction with syndecan-1 is needed in order to achieve full adhesion and integrin signaling, as well for cortical actin to organize correctly when cells are plated on collagen. On the other hand, syndecan-1 is not able to mediate cell adhesion alone (Vuoriluoto et al. 2008). In addition, co-operative behavior between integrins αVβ3 (Beauvais and Rapraeger 2004) and αVβ5 (McQuade et al. 2006) (to vitronectin) as well as integrins α6β4 (Ogawa et al. 2007) and α2β1 (Hozumi et al. 2006) (to laminin) with syndecan-1 have been reported. Syndecan-4 seems to be involved when α5β1 binds to fibronectin, (Bloom et al. 1999). A direct interaction

between the syndecan-4 core protein and β1-integrins has also been shown (Whiteford and Couchman 2006).

Syndecans have been linked to inflammation and cancer, where they act as regulators of cell adhesion and migration. Interestingly, syndecan-1 has been shown to be a prognostic marker for different cancers. In some cancer types a high level and in other cancers a low level of syndecan-1 predicts an aggressive disease (Couchman et al. 2015). The knockout phenotype showed that syndecan-4 participates in wound healing and angiogenesis in granulation tissue *in vivo* (Echtermeyer et al. 2001). Syndecan-1 knockout mice also have problems with wound healing (Pal-Ghosh et al. 2008). In contrast to these repair phenotypes, the migration and survival of neuronal cells is defective in the syndecan-3 knockout mice (Hienola et al. 2006 and Paveliev et al. 2008). A syndecan-2 knockout mouse has not been reported. Although syndecans are important in cell-extracellular matrix (ECM) interactions, they are thought to be more like co-receptors. The reason for this is the fact that many integrin null mice have severe phenotypes compared to the relative mild phenotypes of syndecan knockouts (Couchman et al. 2015).

1.2. Transforming growth factor β

The transforming growth factor β (TGF- β) superfamily can be considered one of the most important growth factor families. It consists of 42 different growth factors or cytokines such as TGF-β1, -2, and -3, myostatin, anti-mullerian hormone (AMH) and bone morphogenic proteins (BMPs) (Massagué and Gomis 2006). The critical roles of the TGF- β family members have been demonstrated under numerous physiological and pathological conditions, including the regulation of stem cell differentiation (Xie and Spradling 1998), as well as in diseases like cancer and fibrosis (Margadant and Sonnenberg 2010). In addition, TGF- β signaling is important in the regulation of immune cells and its main function in the immune system is to maintain tolerance. Thus, TGF- β signaling is mainly inhibitory, but it can also be the opposite. TGF- β regulates the differentiation of effector cells, the proliferation of B- and T-lymphocytes, the secretion of the effector cytokines as well as the function of macrophages, natural killer cells, and dendritic cells. TGF- β is an important player in many autoimmune disorders (Li et al. 2006, Yoshimura et al. 2010).

TGF- β1, TGF- β2, and TGF- β3 form their own structural and functional subgroup (Massagué 1990). Cells secrete TGF- β in its latent form. The active growth factor is located in the carboxy-terminus of the polypeptide. The N-terminal part of pro-TGF-β is called latency associated peptide (LAP) (Worthington et al. 2011). After the synthesis of the pro-TGF-β polypeptide, two pro-TGF-β polypeptide chains form a dimeric structure and furin class proteases cleave the polypeptide chain between LAP and the active TGF- β in the trans-Golgi network (Dubois et al. 2001). Although LAP and the active TGF- β are no longer covalently linked, they still form a complex and LAP prevents the binding of the active TGF- β to its receptor. The active-TGF- β-LAP-

complex is called the small latent complex (SLC) (Fig. 21). The crystal structure of the SLC (porcine proTGF- β) is ring-shaped with a long structurally loose latency lasso covering the amino acids critical for the interaction between the active TGF- β and the TGF- β receptor (Shi et al. 2011).

However, cells do not usually secrete the SLC but the so called large latent complex (LLC) (Fig. 21). In LLC, LAP is attached to the latent TGF- β binding protein (LTBP) by disulfide bonds in the endoplasmic reticulum. There are four different LTBPs (LTBP-1-LTBP-4), but LTBP-2 does not bind to any TGF- βs, and LTBP-4 binds only to TGF- β1-LAP. LTBPs target TGF-βs to the extracellular matrix. LTBPs associate with fibrillin microfibrils by binding directly to fibrillin. Alternatively, they can mediate binding to other proteins of the extracellular matrix, like fibulins or fibronectin (Hyytiäinen et al. 2004, Robertson et al. 2015). LTBPs have shown to be needed for the activation of TGF- β from the latent complex (Annes et al. 2004).

1.2.1. Integrin-mediated activation of transforming growth factor β

The αV integrins are involved in the processes leading to TGF- β activation (Munger et al. 1999). It has been been shown, that integrins αVβ1 (Reed et al. 2015), αVβ3, αVβ5, αVβ6, and αVβ8 can activate latent TGF- β. It cannot be activated by α5β1, α8β1, or αIIbβ3 although they are also able to bind to LAP (Wipff and Hinz 2008). The high affinity integrin binding is mediated by the RGD of β1-LAP and β3-LAP. β2-LAP lacks the RGD sequence and it is not thought to be activated by integrins, althought integrin αVβ6 can bind to β2-LAP with lower affinity (Dong et al. 2014).

Some integrins, such as αVβ8, activate TGF- β in collaboration with MT-1 MMP or other matrix metalloproteinases. Integrin αVβ6 and some other integrins seem to activate latent TGF- β directly by allocating force to the TGF- β complex, which changes the conformation of the latent complex and leads to the release of active TGF- β (Wipff and Hinz 2008; Buscemi et al. 2011). The force mechanism is supported by the following evidence:

1) Integrins cannot activate TGF- β from the SLC. This was shown to be the case when soluble SLC was added to a co-culture of reporter cells (Annes et al. 2004). In addition, soluble recombinant αVβ6 cannot activate latent TGF- β (Shi et al. 2011).

2) The binding of LLC to the ECM is needed for TGF- β activation. Mutations in the region that is important for the binding of LTBP1 to the extracellular matrix inhibit the activation of TGF- β (Annes et al. 2004). This is also the case when the covalent association between LAP and LTBP is broken (Yoshinaga et al. 2008).

3) The stiffness of the matrix can affect the activation of TGF- β (Hinz 2009).

4) The disruption of the actin cytoskeleton prevents the activation of TGF- β (Wipff and Hinz 2008).

5) An experiment that was performed with magnetic beads coated with integrin αVβ6 directly showed that the latent TGF- β become activated by the mechanical force (Buscemi et al. 2011).

The integrin-mediated activation of TGF- β has been shown to have an important role *in vivo*. The most remarkable example is the phenotype of the $Tgfb1^{RGE/RGE}$ transgenic mouse (Yang et al. 2007). In this mouse, the binding of integrin TGF- β1-LAP is prevented by mutating the RGD motif to RGE. The phenotype of this mouse is similar to that of the $Tgfb1^{-/-}$ mouse, which completely lacks TGF- β1. The phenotype of both mice includes multiorgan inflammation, lack of Langerhans cells as well as problems in angiogenesis. The phenotype of $Tgfb1^{RGE/GRE}$ is likely caused by the prevention of integrin-mediated activation, but not changes in the biosynthesis of TGF- β1 or the capability of LAP to inactivate TGF- β1 (Yang et al. 2007). The double knockout or pharmacological inhibition of integrins β6 and β8 generates the phenotype of $Tgfb1^{-/-}$ and $Tgfb3^{-/-}$ mice (Aluwihare et al. 2009), which confirms the essential role of integrin in the activation of TGF- β *in vivo*.

The interplay between integrins and TGF- β is far more complex than activation only. For example, TGF- β regulates the expression of integrins and integrin ligands (Ignotz et al. 1989).

1.2.2. Integrin independent activation mechanisms of transforming growth factor β

The rupture of the interaction between active TGF- β and LAP by different factors leads to the activation of TGF- β. Next, some physiological activation mechanisms are presented.

1.2.2.1. Physicochemical activation mechanisms

Very low (pH 1.5) or high (pH 12) pH activates latent TGF- β completely (Lyons et al. 1988). Low pH activates TGF- β *in vivo* at least under acidic conditions by osteoclasts (Pfeilschifter et al. 1990). Besides pH, treatments with chaotropic agents or heat-treatments also lead to the activation of TGF- β (Huber et al. 1991).

1.2.2.2. Redox-mediated activation

The treatment of latent TGF- β with reactive oxygen species causes its activation (Barcellos-Hoff et al. 1996) because the oxidized LAP cannot maintain the structure of

the latent complex. The activation is specific for TGF- β1, and Met253 in the TGF- β1-LAP has an important role in the process (Jobling et al. 2006).

1.2.2.3. Binding-mediated activation

Trombospondin-1, a glycoprotein in the plasma and the extracellular matrix, can activate TGF- β via direct binding and the induction of structural changes (Hyytiäinen et al. 2004). The binding of the mannose-6-phosphate receptor can also activate TGF-β (Dennis and Rifkin 1991).

1.2.2.4. Protease and glycoside activation

Although thrombin, elastin, cathepsin, plasmin and some other proteases can activate TGF-β *in vitro* (Wipfff and Hinz 2008), the role of the protease-mediated activation of TGF- β is unclear *in vivo*. For example, the mouse phenotypes of plasminogen null and TGF- β1 null mice do not share obvious similarities (Hyytiäinen et al. 2004). Glycosides can activate TGF-β via removing glycosylation (Miyazono and Heldin, 1989). However, also unglycosylated TGF-β-LAP can inhibit the function of active TGF-β1 (Yang et al. 1997).

1.2.3. Transforming growth factor β signaling

There are three cell surface receptors for TGF- β, TGF- βRI-III. TGF–βRIII is a proteoglycan and its function is to harvest the TGF- β protein but it is not a signaling TGF- β receptor (López-Casillas et al. 1993; Massagué 1996). TGF–βRIII is needed especially in the case of TGF- β2 that binds to other TGF- β receptors with very low affinity (Bilandzic and Stenvers 2011). TGF-βRI and TGF-βRII are signaling receptors (Laiho et al. 1990). The active TGF- β binds first to TGF- βRII, which leads to the formation of a bidimeric complex consisting of two TGF- βRIs and two TGF- βRIIs. TGF-βRII phosphorylates certain serine and threonine residues in TGF- βRI. As a consequence, TGF- βRI becomes active (Massagué and Gomis 2006). Activated TGF-βRI phosphorylates the carboxyterminus of the Smad2/3 transcription factor in the canonical TGF- β pathway. After its release from the receptor complex, Smad2/3 binds to Smad4 and translocates into the nucleus (Massagué et al. 2005). The process is regulated by many other phosphatase and ubiquitine ligases as well as other activatory or inhibitory signals. The Smad-4 indepent non-canonical TGF- β pathway has also been shown to play an essential role in some physiological processes like hematopoiesis (Massagué and Gomis 2006, He et al. 2006). The components of TGF- β signaling interact with many other transcription factors like HOX, RUNX, and E2F. This enables the simultaneous activation of some genes and the inactivation of others. The stimulation of TGF- β leads to the altered expression up to a couple of hundred of genes (Massagué and Gomis 2006).

1.3. Citrullination

1.3.1. Citrullination and PAD enzymes

Citrulline is a naturally occurring amino acid originally found in the watermelon, *Citrullus lanatus* (Fearon 1939). Citrulline does not have a genetic code, rather it becomes part of proteins through a posttranslational modification. Citrullination, or peptidyl deimination, is an enzymatic process catalyzed by peptidyl arginine deiminase (PAD, EC 3.5.3.15, Fujisaki and Sugawara 1981). The amino acid residue that becomes modified is arginine and the change in mass, caused by the reaction, is 0.987 Da (György et al. 2006) (Fig. 2). Thus, citrullination is a relatively minor change, but it leads to the neutralization of the positively-charged arginine. In addition to being functionally important (Patthy and Smith 1975) arginine residues are stabilizing elements in proteins (Mrabet et al. 1992). Therefore, the impact of citrullination on protein structure and function can be considerable. Citrullination may disrupt protein structures by breaking salt bridges within and between polypeptide chains. In some globular proteins, the citrullination of 10 % of the protein's arginines can lead to the nearly complete unfolding of its three-dimensional structure (Tarcsa et al. 1996). A shift in the protein's isoelectric point is an obvious consequence of protein citrullination (Takahara et al. 1985).

While catalyzing a citrullination reaction, a cysteine residue in the active site of the PAD enzyme attacks a guanidino group of the target arginine, which leads to the release of an amino group and to the formation of a tetrahedrical intermediate. A nucleophilic attack by water is required for the formation of the peptidyl citrulline (Arita et al. 2004). The PAD enzymes need Ca^{2+} as a co-factor (Fujisaki and Sugawara 1981). The calcium ion does not participate in the catalysis, but is needed for the conformational activation of the enzyme (Arita et al. 2004). Under normal physiological conditions, the calcium concentration is usually small enough to prevent the activation of PAD, and thus allows the regulation of the enzymes (Takahara et al. 1986, György et al. 2006). Some of the PAD ligands, like trichohyalin, are calcium binding proteins, which may provide a source of calcium ions for the PADs (Vossenaar et al. 2003).

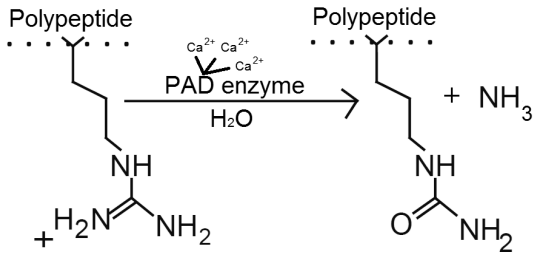


Figure 2. Mammalian PAD enzyme catalyses the conversion of peptidyl arginine to peptidyl citrulline (Fujisaki and Sugawara 1981). Ca^{2+} is needed for the activation of PAD (Arita et al. 2014).

Humans have five different PAD enzymes in (PAD1, PAD2, PAD3, PAD4 and PAD6). The biological functions and expression patterns of the PAD isoforms vary (Vossenaar et al. 2003). PAD1 is expressed in the epidermis, where it citrullinates its most important physiological substrates, filaggrin and keratin 1 during the maturation and cornification of the epidermis (Nachat et al. 2005). In the case of filaggrin, citrullination seems to make it an easier target for some proteases, which enables the formation of macrofibrils. PAD1 is also expressed in the uterus (Vossenaar et al. 2003). Like PAD1, PAD3 is also expressed in the skin, but the two enzymes are expressed in different locations of the skin. The natural ligand for PAD3 seems to be trichohyalin, which is citrullinated by PAD3 in the medulla and in Henle's layer (Nachat et al. 2005).

PAD2 is the most broadly expressed PAD enzyme. Besides the skin, it is also expressed in, for example, the nervous, digestive and hematological systems (Vossenaar et al. 2003). An important physiological substrate for PAD2 is myelin basic protein (MBP) in the central nervous system (Finch et al. 1971, Pritzker et al. 2000). The citrullination of MBP leads to an increase in its proteolytic degration by cathepsin D. The percentage of citrullinated MBP correlates with the plasticity of human brain development. Citrullination reaches is maximum levels in the brain at four year's of age (Moscarello et al. 1994 and György et al. 2006).

PAD4 (or PADV) is important for early embryonic development and in the immune system. PAD4 is the only PAD enzyme that has a nuclear localization signal peptide (Vossenaar et al. 2003). In the nucleus, PAD4 modifies histones and represses transcription by antagonizing arginine methylation (Cuthbert et al. 2004). In addition, the citrullination of histone H1 by PAD4 is needed for the generation of normal ground state pluripotency in mouse embryonic stem cells *in vivo* (Christophorou et al. 2014). An other major function of PAD4 is related to inflammation-activated myeloid cells. Citrullination can cause the decondensation of chromatin that is released from the cells for the formation of neutrophil extracellular traps (NETs) (Wang et al. 2009). Another function of PAD4 seems to be the regulation of intracellular signaling pathways. Glycogen synthase kinase-3β (GSK3β) is a target of PAD4 mediated citrullination *in vivo*. The citrullination of GSK3β regulates TGF-β signaling and can induce an epithelial-to-mesenchymal transition in breast cancer cells (Stadler et al. 2013).

PAD6 is mainly expressed in egg cells and during early embryonic development (Vossenaar et al. 2003). PAD6 null female, but not male, mice are infertile (Esposito et al. 2007). The function of PAD6 seems to be linked to the reorganization of intermediate filaments, and it has been shown to interact with and regulate the formation of cytoplasmic lattices, a structure found in oocytes (Kan et al. 2011).

Interestingly, the prokaryotic pathogen *Porphymonas gingivalis* also has a PAD enzyme (PPAD) (McGraw et al 1999). There is no sequence homology between mammalian PADs and PPAD (Mangat et al. 2010). Contrary to the mammalian PAD

enzymes, PPAD does not need calcium and it can also citrullinate free arginine (McGraw et al 1999). Still, the mechanism of catalysis is dependent on a cysteine-histidine-asparagine triad that is also be found in human PAD1, PAD2, PAD3, and PAD4. PPAD prefers C-terminal arginines as its targets for citrullination (Goulas et al. 2015). The role of PPAD in *P. gingivalis* is unclear, but it may modulate the growth environment of the bacteria in the gingival pocket (Mangat et al. 2010).

1.3.1.1. Citrullination of extracellular matrix proteins

PADs lack a secretory signal peptide typical for secretory proteins. Thus, their major known physiological targets, like histones and keratins, are intracellular proteins (Vossenaar et al. 2003). However, histological studies on collagen induced arthritis in rats revealed that the citrullination is mainly localized to the extracellular space (Lundberg et al. 2005). The result was interesting, but then it was not known whether the calcium concentration and other conditions allow the extracellular activation of the PAD enzyme. Later it was shown that the calcium concentration was high enough for PAD activation in the synovial fluids of patients with joint inflammation (Damgaard et al. 2014). In addition, the calcium concentration increase due to inflammation related apoptosis or necrosis (Uysal et al. 2010). Active PAD4 and PAD2 can be detected in the synovial fluids of arthritis patients (Damgaard et al. 2014) as well as in the bronchoalveolar lavage fluid (Damgaard et al. 2015) derived from the lungs of tobacco smokers. PAD2 and PAD4 are the only haplotypes of the peptidyl arginine iminases that are expressed in the synovium (Foulquier et al. 2007). Activated leukocytes and hyperplastic synoviocytes are the primary sources of PADs in inflamed joints.

The mechanism, by which the active PADs are released from cells into the extracellular space as a consequence of inflammation, is not completely understood (Spengler et al. 2015). Increased apoptosis and necrosis in the synovium are among the mechanisms that have been suggested (Uysal et al. 2010). The most interesting hypotheses for explaining extracellular citrullination are related to the formation of neutrophil extracellular traps (NETosis) (Spengler et al. 2015) or an immune-mediated membranolytic pathway, the membrane attacking complex (MAC) /perforin pathway (Romero et al. 2013). In NETosis, PAD4 is needed for chromatin decondensation and it can be released into the extracellular space together with the traps (Spengler et al. 2015). It has been shown that PAD4 interacts with the protein-DNA complexes of the NETs and the citrullination of RA autoantigen related proteins occurs extracellularly. Unlike NETosis, the MAC/perforin pathway has been shown to activate intracellular hypercitrullination (Romero et al. 2013). Here, citrullination occurs in the intracellular space, but also extracellular proteins, like collagen, become citrullinated before secretion or necrotic release. Both of these mechanisms are relevant for the pathogenesis of RA.

The extracellular matrix proteins that have been reported to undergo citrullination in joint inflammations are collagen I (Romero et al. 2013) and II (Uysal et al. 2009), fibronectin (Chang et al. 2005), fibrinogen (Masson-Bessière et al. 2001), periostin and proteoglycan 4 (van Beers et al. 2013). Fibrin is known to be citrullinated in the synovium of RA patients as well as in animal models of arthritis (Vossenaar et al. 2003B). Like fibrinogen, citrullinated fibronectin can be found in the RA synovium (Chang et al. 2005). The citrullination of collagen II has been detected in RA as well as in osteoarthritis (Uysal et al. 2009). In addition to possibly being autoantigenic, the citrullinated extracellular proteins may behave differently than their unmodified counterparts. Citrullinated fibrinogen cannot be polymerized normally by thrombin mediated catalysis (Nakayama-Hamada et al. 2008). On the other hand, it has been suggested that the citrullination of fibronectin increases synovial proteolysis by preventing the inhibitory interaction between fibronectin and the metalloproteinase ADAMTS4 (Yan et al. 2013). In addition, adhesion and migration as well as integrin signaling are decreased in fibroblasts, such as synoviocytes, when the cells are plated on citrullinated fibronectin (Shelef et al. 2012).

1.3.2. Citrullination in rheumatoid arthritis

1.3.2.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease that leads to the dispruption of the joint cartilage. The clinical diagnosis is based on the American College of Rheumatology (ACR) classification criteria (Uysal et al. 2010). The pathogenesis of RA and many other autoimmune diseases can be divided to the three stages. During the first stage, some environmental factors trigger an autoimmune response; in the second stage, the joints are affected by the immune response; and in the third stage, the inflammation becomes chronic (Holmdahl et al. 2014). Previously, RA was a highly disabling disorder, which could not be treated effectively. Today, a group of drugs, called disease-modifying antirheumatic drugs (DMARDs) consisting of general anti-inflammatory drugs like methotrexate and TNF-α blockers, is available. DMARDs are effective in the treatment of erosive RA (Tracey et al. 2008).

1.3.2.2. Autoantibodies against citrullinated protein epitopes

The first clues to imply that citrullination is an important phenomenon in the pathology of rheumatoid arthritis (RA) came from research into autoantibodies. The so called anti-keratin antibodies (also known as the anti-perinuclear factor) (Sebbag et al 1995), that have been used for diagnosing rheumatoid arthritis, actually recognized the protein epitopes in which arginine residues were converted to citrulline (Schellekens et al. 1998). The autoantibodies, known as anti-citrullinated protein antibodies (ACPA), are present in patients long before the clinical onset of the RA (Nielen et al. 2004). The finding allowed the development of synthetic citrullinated peptide based assays for more specific clinical tests for the detection of RA

(Schellekens et al. 2000). With a positive ACPA test result the probability of developing RA is about 98 %. On the other hand, about 70 % of all RA patients are positive for ACPA (van Venrooij at al. 2011).

In addition to their important diagnostical value, the RA autoantibodies have been shown to have an important pathophysical role in arthritis (Uysal et al. 2009). In recent studies, ACPAs have shown to recognize citrullinated filaggrin (Union et al. 2002), fibronectin (van Beers et al. 2012), vimentin (Vossenaar et al. 2004), α-enolase (Kinloch et al. 2006), and collagen II (Burkhardt et al. 2005), for example. Some of the ACPAs, like autoantibodies against citrullinated and unmodified collagen II, can mediate arthritis by binding to cartilage (Uysal et al. 2009, Haag et al. 2014). However, some autoantibodies can also protect against joint erosion, which may explain why they can be detected a long time before the clinical symptoms (Croxford et al. 2010).

ACPAs tend cross-react with unmodified target molecules (Uysal et al. 2010), which might explain the epidemiological link between RA and periodontitis. As was described above, a gingival pathogen called *Porphymonas gingivalis* has its own enzyme for citrullination, PPAD (Wegner et al. 2010). Thus, autoantibodies may form after citrullination by PPAD locally in gingiva, and the antibodies can then cross-react with unmodified epitopes elsewhere in the body. *P. gingivalis* has been shown to increase inflammation in animal models of arthritis (Maresz et al. 2013), but the significance of this observation for the human disease remain to be investigated. Alternatively, the formation of cross-reactive autoantibodies can be triggered in the lungs of tobacco smokers, which could also explain the connection between smoking and rheumatoid arthritis (Klareskog et al. 2006).

Interestingly, the presence of citrullinated proteins has been shown in different types of inflammation, although ACPAs are highly specific for RA (Vossenaar et al. 2004). Immunostaining of citrulline residues and microscopic examinations of inflamed human tissues have shown that citrullination occurs in muscles affected by myositis, in chronically inflamed tonsils as well as in synovial biopsy samples from rheumatoid arthritis patients, but it is rarely detected in normal tissues (Makrygiannakis et al. 2006). This indicates that citrullination is a common consequence of inflammation. However, citrullinated proteins are more immunogenic than unmodified proteins and the induction of collagen induced arthritis (CIA) by citrullinated collagen II is increased when compared to unmodified collagen II (Lundberg et al. 2005).

Rheumatoid arthritis patients can also have autoantibodies other than ACPA. Interestingly, patients who have autoantibodies that cross-react with PAD3 and PAD4 enzymes show more signs of erosive arthritis than RA patients, who are negative for this subset of antibodies. These antibodies make the PAD4 enzyme more sensitive to its co-factor Ca^{2+}, which may boost the enzyme's activity in the synovium *in vivo*. Citrullination has many intracellular functions that can have an impact on inflammation, but the finding of PAD4-PAD3 antibodies suggests that extracellular

citrullination has an important role in the progression of rheumatoid arthritis (Darrah et al. 2013).

1.3.2.3. Citrullination and genetics

In addition to serological studies, human genetics has revealed a connection between citrullination and RA. A certain haplotype of the PAD4 gene (*PADI*) located in the CHR1p36 area of the locus D1S228 has been shown to be a risk factor for rheumatoid arthritis in Asian populations (Suzuki et al. 2003). The known single nucleotide polymorphism (SNP) seems to be functional at the molecular and systemic level. At the molecular level the SNP can stabilize the messenger RNA and possible lead to the higher amount of PAD4. At a systemic level, it increases the formation of the ACPAs (Horikoshi et al. 2011). However, the role of *PADI4* as a genetic risk factor for RA has not been confirmed by all population studies (Hou et al. 2013). SNPs in the gene of PAD2 have also been suggested to significantly associate with RA (Chang et al. 2013).

Another genetic risk factor for RA, which is related to citrullination, is a polymorphism in PTPN22, a protein tyrosine phosphatase expressed in hematopoietic tissues (Chang et al. 2015). The C-to-T SNP at position 1858 and the following mutation R620W have functional consequences. PTPN22 has been shown to directly interact with PAD4 and the mutation in PTPN22 inhibits this interaction, but does not affect the phosphatase activity. The disruption of the PTPN22-PAD4 interaction leads to a higher level of citrullination and spontaneous formation of neutrophil extracellular traps. It is still unclear if the the autoimmune reaction is primarily due to the increase in citrullination or the increase in leukocyte activation. The R620W polymorphism is the strongest genetic risk allele for RA excluding the HLA variants (Stanford and Bottini 2014).

1.3.3. Citrullination in other diseases

In addition to joint inflammation, citrullination is linked to many other diseases that have an immunological component. In multiple sclerosis (MS-disease), the amount of citrullination in myelin basic protein (MBP) increases, which affects its ability to aggregate and makes it more vulnerable to cathepsin D mediated proteolysis (Cao et al. 1999). Protein citrullination in the hippocampus area of the brain has been reported to increase in Alzheimer's disease (Ishigami et al. 2005). Unlike in neurological diseases, citrullination is decreased in skin psoriasis. The citrullination of keratin 1, which is a normal physiological target for PAD, is decreased in psoriasis (Ishida-Yamamoto et al. 2000). The expression of PAD4 is increased in many malignant tumours like breast and lung adenocarcinomas. PAD4 has also been detected in the blood of cancer patients. Interestingly, benign tumours do not express PAD4 more than healthy tissues. Hematopoietic CD34+ -cells seems to be the main source of PAD4 in carcinomas (Chang and Han 2006).

1.3.4. Inhibition of citrullination as a therapeutic approach

During the last decade, small molecule inhibitors against PADs, especially against PAD2 and PAD4, have shown promise as a therapeutic strategy. Inhibitors, like N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide (Cl-amidine), against the PAD4 enzyme seem to ameliorate joint inflammation in some animal models of arthritis (Willis et al. 2011). Cl-amidine has no effect on arthritis induced by an antibody against collagen, but it reduces the severity of arthritis induced by collagen, indicating that the mechanism is independent of the effectory pathway. The PAD4 inhibitors are potential drugs also for treating other diseases. It has been shown in animal models that the inhibitors are effective at least in the treatment of hemorrhagic shock (He et al. 2015), kidney ischemia-reperfusion injury (Ham et al. 2014), hypoxic ischemic insult in neonates (Lange et al. 2014), atherosclerosis (Knight et al. 2014), and multiple sclerosis (Moscarello et al. 2013). The therapeutic mechanism in all these cases can be the prevention of inflammation, but also other mechanisms for disease prevention may exist.

2. AIMS

The aim of this Ph.D. project was to study integrin-ligand interactions and their molecular mechanisms in different biologically interesting contexts. The function of integrins in these cases is incompletely understood. The work was carried out in order to increase our knowledge about virus infections, thrombus formation, cancer angiogenesis, and joint inflammation, like in rheumatoid arthritis. The specific aims and background of each subproject are described below.

2.1. Echovirus 1 binding to integrin α2β1 (Study I)

Echovirus 1 (EV1) uses integrin α2β1 for its entry into cells (Bergelson et al. 1992). It is also known that the echovirus binding site is in the integrin αI domain, which is responsible for binding to the native integrin ligands, such as collagen (Bergelson 1994). The mutation E318W in the integrin α2I domain leads its conformational activation and an increased affinity to collagen (Aquilina at al. 2002). In contrast, the mutation E336A seems to inactivate integrin and make binding to its ligands weaker (Connors et al. 2007). These mutations have been thought to mimic the physiological conformational states of the integrin, which are regulated by outside-in and inside-out -signaling. However, it is not known if the virus binds to the integrin by a mechanism similar to that of the ligands of the extracellular matrix. The aim of this subproject was to study how the conformational states of integrin α2β1 affect the binding of EV1.

2.2. Integrin α2β1 mediated adhesion under flow conditions (Study II)

The conventional model for the integrin-ligand interaction in αI-domain containing integrins under flow conditions is based on leukocyte integrins. In this model, the main idea is that integrins are in an inactive conformation in the blood stream, which prevents binding to the vascular endothelia or soluble ligands. After pre-activation, mediated by inside-out signaling, the integrins become conformationally activated and can bind to their ligands (Arnaout et al. 2005, Luo et al. 2007). However, it is not known if integrin α2β1, another important platelet integrin, binds to its ligand via the same mechanism. In this subproject, the aim was to study how the conformational states of integrin α2β1 affect ligand binding under flow conditions.

2.3. The interplay between histidine-rich glycoprotein and integrins (Study III)

Histidine-rich glycoprotein (HRG) is a common plasma protein and an angiogenesis inhibitor (Olsson et al. 2004). Although HRG has been shown to inhibit the adhesion of leukocytes, the mechanism of this inhibition is unclear (Olsen et al. 1996). Furthermore, it is not completely known if HRG can also affect the adhesion of endothelial cells. Understanding this could shed light on the mechanism of angiogenesis inhibition. In this subproject, the aim was to to stude the effects of HRG on the adhesion and migration of endothelial cells. In addition, the possible interplay between integrins and HRG was examined.

2.4. Extracellular citrullination (Studies IV-VI)

The citrullination of ECM proteins as well as extracellular PAD activity (peptidyl arginine deiminase) have been found to occur due to inflammation (Uysal et al. 2009, van Beers et al. 2013, Damgaard et al. 2014). The role of citrullination in the generation of autoantibodies specific for rheumatoid arthritis has been the target of extensive research (van Venrooij et al. 2011), but the effects of citrullination itself on extracellular proteins has received less attention. The ECM has three different functions: formation of tissue structure, attachment to cells, and deposition of growth factors (Hynes 2009). The aim of his subproject was to demonstrate how extracellular citrullination affects the functions of the ECM. The aims are elaborated below:

1) Collagen II is known to be citrullinated *in vivo* in joint inflammation (Uysal et al. 2009). Our aim was to study how different collagen receptors recognize the citrullinated collagen. In addition, the adhesion of fibroblast like synoviocytes to citrullinated collagen was tested. (IV)

2) To demonstrate how extracellular citrullination affects the function of ECM associated growth factors, we focused on TGF-β, which is an important growth factor relevant for inflammation and located in the ECM. The effects of extracellular citrullination on the integrin-mediated activation of TGF-β and on the binding of TGF-β to its receptor were studied. (V)

3) The prevalence of extracellular citrullination as well as the arginine residues that can become citrullinated are incompletely known *in vivo*. The idea of this subproject was to find out whether functional arginine residues, like integrin binding sites, become citrullinated *in vivo*. For this purpose, synovial fluids from patients with chronic joint inflammation were collected and analyzed by mass spectrometry for the detection of citrullinated extracellular proteins. (VI)

3. METHODS

The focus of this section is on the methods that were used in making the main discoveries described in this thesis. A full description of the materials and methods can be found in the original studies I-VI.

3.1. Cells and cell lines (Studies I -VI)

C2C12. C2C12 is a mouse satellite cell line. The cells do not express collagen receptor integrins on their surface (Tiger et al. 2001). Thus, the human collagen receptor integrins can be transfected into C2C12 cells and studied individually.

CHO. The cells of the Chinese hamster ovary cell line do not express collagen receptor integrins on their surface. Thus, the human collagen receptor integrins can be transfected into CHO cells and studied individually (Käpylä et al. 2004). The cell line transfectants are widely used in our cell adhesion studies.

CHO-LTBP1. CHO-LTBP1 transfectants were used to generate a TGF-β-rich extracellular matrix. TGF-β is secreted in the LLC and LTBP1 is important for its deposition into the ECM (Unsöld et al. 2001).

Fibroblast-like synoviocytes. Fibroblast-like synoviocytes are primary cells isolated from the joints of osteoarthritis patients. These cells were used as a model for human synovial fibroblast in the collagen study.

HaCaT. HaCaT cells are immortalized human keratinocytes derived form skin. They express αVβ6 integrin on their surface (Koivisto et al. 1999). Latent TGF-β can be activated by αVβ6 integrin, and for this reason the HaCaT cells were used in the model for the activation of latent TGF-β from the ECM.

SaOS-2. Saos-2 is a cell line derived from human osteosarcoma. Saos-2 cells have osteoblastic properties (Rodan et al. 1987). For this reason, they were used in the study on collagen citrullination.

Mesenchymal Stem Cells. Human mesenchymal cells are isolated from bone marrow. These cells were used because they are important in the repair of cartilage in arthritis (Marinova-Mutafchieva et al. 2002), but also because the integrins α2β1 and α11β1 have and important role in promoting their survival (Popov et al. 2007).

MLEC-PAI-1/Lu reporter cells. These mink lung epithelial cell transfectants are reporter cells for TGF-β (Abe et al. 1994).

HUVECs. HUVECs are human umbilical vein endothelial cells that are typically used as a cell culture model for human vascular endothelial cells (Jaffe et al. 1973).

3.2. Mutagenesis of full length integrin α2 (Study I)

To create the mutations in the full length α2 subunit, the α2-pAWneo vector was restricted with BamHI and the restriction fragment was ligated into the pGEX-2T vector. This ca 2 kbp cassette included the integrin α2 bases 1-1815 and a small piece of vector. The mutations were prepared using the QuikChange mutagenesis method (Stratagene). The method is based on the PCR of amplification of the plasmid using mismatching primers with the mutation. The template plasmid can be destroyed by DpnI-digestion that cleaves only the methylated plasmid but not the PCR product. After the digestion, *E. coli* cells were transformed with the PCR reaction. Plasmid was purified from E. coli using standard methods and the mutation was confirmed by Sanger sequencing. The cassette was restricted-ligated back into the α2-pAWneo vector (BamHI-restriction) or α2-pcDNA3 (AsiSI and BamHI). The CHO-K2 cell line was transfected with the plasmid using the FuGENE® HD Transfection Reagent (Promega) and a standard protocol.

3.3. Cell adhesion measurements (Studies I-VI)

Cell adhesion was assayed based on two different methods: xCELLigence technology and a plate and wash –assay. The xCELLigence technology is based on changes in impedance caused by the attachment of cells to a plastic surface covered by a gold grid. 96-well E-Plate 96 (ACEA Biosciences) wells were coated by diluting the ligands (3-5 μg/cm^2) in PBS and incubating them in the wells overnight in 4 °C. The wells were blocked for 1 h at RT with BSA (1 mg/ml). The cells were trypsinized or detached with 5 mM EDTA, washed with PBS, and seeded into the wells with cell culture medium. In some cases, the cells were pre-treated before seeding and the medium could be supplemented with serum or $MgCl_2$. A typical cell amount varied from 15 000/well to 20 000 / well. Cell spreading was measured with the xCELLigence system (Roche Applied Science).

The plate and wash -assay was performed largely as described above, but the detection was based on the tetrazolium salt WST-1 reagent (Roche) that is processed into the soluble formazan by metabolically active cells. Formazan can be detected spectrophotometrically (absorbance at 420-480 nm). The 96-well plates (cell culture plastic) were coated with ligands (diluted in PBS, 5-20 μg/cm^2) at 4 °C overnight. After blocking the plates with BSA, 200 000 detached cells (the substantial amount of cells was used for covering the entire plasctic surface with cells and for reaching the maximal signal) were seeded into the wells and incubated for 15 -30 min in a cell incubator (+37 °C, 5 % CO_2). The wells were washed three times with PBS to remove

any cells that had not adhered. The number of attached cells was quantified with the WST-1 reagent.

3.4. Protein binding measurements (Studies I-VI)

Protein binding measurements were based on a solid-phase binding assay. In this assay type, 96-plate wells are coated with the ligand or the receptor. The interacting proteins are incubated in the well. The wells are washed and the bound protein is detected with labeled antibodies. Typically, the 96 well plate wells were coated overnight with the ligand at +4°C. The wells were blocked with BSA or Delfia diluent (PerkinElmer) (1 mg/ml, RT, 1h) and the receptors were added and left to bind (1 h, RT) in the Delfia assay buffer (PerkinElmer). After washing the wells, the primary antibody was added. After washing, the Eu-labeled (PerkinElmer) secondary antibody was added. After a 1 h incubation, the wells were washed and enhancement solution was added. The signal was detected with time-resolved fluorescence spectrophotometry (Victor^3 multilabel counter; PerkinElmer). A typical washing step was three-times with PBS.

3.5. Cell adhesion measurements under flow (Study II)

The cell adhesion measurement under flow was performed with the Cellix microfluidic platform (Cellix) that is a combined fluorescence microscopy and flow chamber instrument. Whole blood or CHO cells were stained with 1 M 3,3-dihexyloxacarbocyanine iodide (Invitrogen). Capillaries were coated with fibrillary collagen (Cellix). Shear rates of 0.01, 90, or 120 dynes / cm^2 were used. The results were analyzed with the DucoCell software (Cellix).

3.6. Functional studies of transforming growth factor β (Study V)

Functional assays for active and latent TGF-β were performed. MLEC-PAI-1/Lu cells were used as reporter cells for TGF-β. These cells had been transfected with a plasmid, where luciferase is under the promoter fragment of plasminogen activator inhibitor-1 (Abe et al. 1994). That promoter is strongly regulated by TGF-β signaling. The ability of active TGF-β1 to induce the signaling was measured by seeding 15 000 MLEC-PAI-1/Lu cells in DMEM supplemented with 10 % FCS. The cells were incubated for 5 h in a cell incubator (+37 °C, 5 % CO_2). Samples were prepared by diluting active TGF-β1 in DMEM supplemented with BSA (0.1 mg/ml). BSA was added because TGF-β1 tends to stick to surfaces. After the incubation, the medium was replaced with the samples. Concentrations from 0.1 ng to 10 ng / ml can be measured with this assay. After a 18 h incubation, the wells were washed with PBS and the cells were lysed with Passive lysis buffer (Promega). The Bright-Glo luciferase reagent (Promega) was added

to the lysates and the luminescence was measured with a Victor3 multilabel counter (PerkinElmer).

The assay, described above, can also be used to study latent soluble TGF-β. Either a recombinant human latent TGF-β1 protein (R&D Systems) or latent TGF-β, produced by human synovial fibroblasts and secreted into the growth medium, were used. The latent complex can be activated by heating (80 °C, 20 min) and measured like active TGF-β1. The latent TGF-β associated with the ECM was studied with the ECM produced by CHO-LTBP1 cells. Those cells had been transfected with the latent TGF-β binding protein 1. The ECM was produced by growing CHO-LTBP1 (50 000 / well) in DMEM supplemented with 10 % FCS (72 h, +37 °C, 5 % CO_2). The cells were removed with 15 mM EDTA in PBS. The amount of activated latent TGF-β was analysed with a co-culture of 20 000 HaCaT cells and 15 000 MLEC-PAI-1/Lu reporter cells. MLEC-PAI-1/Lu cells activate latent TGF-β only slightly. The cells were incubated for 18 h in a cell incubator. The amount of TGF-β was measured with Bright-Glo (Promega) as described above.

3.7. Microscopical and flow cytometric analyses (Studies I-IV)

Cell spreading, attachment, and survival were studied with phase-contrast microscopy and with live-cell imaging with an Axiovert 200M microscope (Zeiss) on cell culture plates. The co-localization of HRG and collagen in tissue sections from a T241 mouse was investigated using standard protocols. The 7 µm tissue sections were fixed with ice-cold methanol. Imaging was performed with a Zeiss LSM710 confocal microscope. Staining for a flow cytometric analysis was done using standard protocols and the analysis was performed using a FACSCalibur instrument (BD Biosciences).

3.8. *In vitro* citrullination (Studies IV-VI)

In vitro citrullination was performed either in-solution or on-surface. Recombinant human PAD4 or PAD2 purified from rabbit skeletal muscle (Sigma-Aldrich) was used at different concentrations (typically from 0.5-4 U/ml). In-solution citrullination was performed by diluting the substrate protein and the PAD enzyme in citrullination buffer (40 mM Tris-HCl, pH 7.4 and 2 mM or 5 mM $CaCl_2$) and by incubating the solution at +37 °C for various time periods. The PAD enzyme becomes inactive after a one-hour treatment at +37 °C, but overnight incubations were also used to reach maximal citrullination. On-surface citrullination was performed as in-solution, but the the bottom of the well was first coated with the substrate. After blocking, the PAD enzyme was added in citrullination buffer. Typically, on-surface citrullination was used in protein binding and cell adhesion measurements.

3.9. Synovial fluids and mass spectrometry (Studies V-VI)

Synovial fluid samples were collected by rheumatologists at the Turku University Hospital. Before freezing, the cells in the synovial fuids were removed by centrifugation (10 min, + 4 °C, 2500 g) and the supernatant was used for further analyses. The laboratory of the University hospital made clinical measurements from separate samples preserved in litium heparin tubes. The PAD activity of synovial fluids was measured with a fibrinogen citrullination assay as described earlier (Damgaard et al. 2014). Briefly, the wells of the assay plate were coated with 0.1 mg/ml fibrinogen in PBS and the samples. The samples were diluted 1/4 with citrullination buffer and incubated in the wells o/n at +37 °C. After the reaction the citrullinated fibrinogen was measured using an anti-citrullinated fibrinogen antibody (Mouse Anti-Citrullinated Fibrinogen (20B2), ModiQuest).

For the mass spectrometric detection of citrullination, albumin and hyaluronate were removed by a single-step heparin-agarose fractionation. Synovial fluid samples were diluted to 4 % with a 10 mM Tris-buffer and heparin agarose. After incubation (+ 4 °C, o/n) and intensive washing, heparin agarose –bound proteins were eluted with 1.5 M NaCl. The eluted proteins were precipitated with six volumes of ice-cold acetone (-20°C, o/n). The mass spectrometry samples were prepared using standard protocols with cysteine reduction followed by a trypsin digestion. The mass spectrometry run was performed with a nanoflow HPLC system (Easy-nLC1000, coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray ionization source. The data was analysed with the Proteome Discoverer software, version 1.4. (Thermo Fischer Scientific) using the Mascot search engine (Matrix Science, version 2.4) against Human UniProtKB sequences. Citrullination was included in the dynamic modifications and their existence was checked manually.

3.10. Structural analysis (Studies I, IV-VI)

Three-dimensional protein structures were obtained from the Protein Data Bank (PDB; www.rcsb.org), the protein models were prepared with the Sybyl (Tripos International), Protein Preparation Wizard panel (maestro version-9.9, Schrödinger suit), and using the OPLS-2005 force field (maestro version-9.9 Schrödinger). Bodil was used for structural analyses and for preparing figures.

4. RESULTS AND DISCUSSION

The essential findings of this Ph.D. thesis are described. Some results and control experiments are excluded from the summary.

4.1. Non-activated integrin α2β1 binds to ligands (Studies I-II)

4.1.1. Echovirus 1 binds to non-activated integrin α2β1 (study I)

The E318W mutation in the α2I-domain has been shown to open the I-domain and is thought to mimic the pre-activated state of the integrin. The open α2I-domain can bind to collagen with a higher affinity as well as recognize more ligands than the closed conformation (Aquilina at al. 2002, Siljander et al. 2004). In contrast, the E336A mutation in the α2 subunit is thought to inactivate the integrin. The glutamate residue 336 is in the interaction surface between the α2 and β1 subunits. The E336A mutation dramatically decreases integrin α2β1-mediated binding to collagen I (Connors et al. 2007).

Like the typical physiological ligands of integrin α2β1, EV1 binds to the α2I-domain (Bergelson et al. 1994). However, in this study we showed that the binding mechanism of EV1 differs critically from that of collagen. To demonstrate this, we transfected Chinese hamster ovary (CHO) cells with the full-length integrin α2 subunit containing either the E336A or the E318W mutation. CHO cells do not express collagen receptors on their surface, which allows the study of the transfected α subunit (β1 is from hamster) without interference from the endogenous collagen receptors (Käpylä et al. 2004). A plate and wash –assay (Fig. 3) showed that the activation of integrin α2β1 (mutation E318W) increases the adhesion of CHO transfectants to collagen I. In contrast, the adhesion of CHO-α2E318W to an EV1 coated surface (Fig. 3B) was decreased compared to CHO- α2. When CHO- α2β1E336A was tested, the result was the complete opposite: non-activated CHO cells transfected with α2E336A bound to EV1 more strongly than did cells transfected with wild type integrin α2, but the binding of non-activated integrin α2β1 to collagen is only residual (Fig. 3A). The binding of EV1 to the non-activated integrin was also tested with recombinant α2I-domains. In addition, EV1 did not activate normal integrin signaling. The molecular mechanism for EV1 endocytosis is described in more detail in study I (see study I).

Binding to the inactive integrin α2β1 is of obvious benefit to EV1. This way, the virus does not have to compete with the integrin's physiological ligands like collagen. In

addition, most of the cell surface integrins seem to be in the non-activated conformation, when they are not bound to a ligand, because activated β1 integrins become endocytosed at high rates (Arjonen et al. 2012). Thus, binding to a non-activated integrin increases the number of binding possibilities for the virus. The binding of integrin α2β1 to collagen triggers the MAP-kinase pathway and the phosphorylation of p38 (Ivaska et al. 1999). When the virus binds to the cell surface it does not activate the phosphorylation of p38 and normal integrin α2 signaling, which might be beneficial for the virus (see study I).

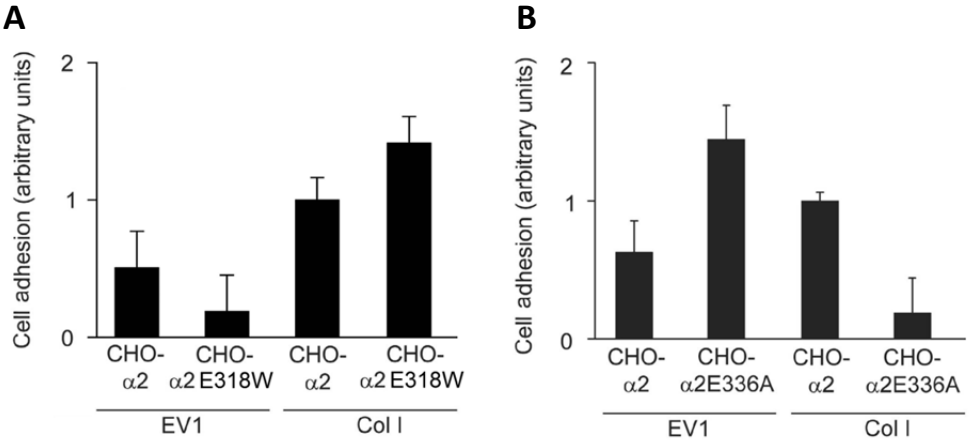


Figure 3. Cell adhesion of CHO transfectants to immobilized collagen I or Echovirus 1 (EV1) measured by a plate and wash -assay. (A) CHO-cells transfected with non-activated integrin α2 (E336A) showed stronger binding to EV1 and weaker binding to collagen 1 (Col1) than CHO cells transfected with wild type α2. (B) CHO-cells transfected with activated integrin α2 (E318A) showed weaker binding to EV1 and stronger binding to Col1 than CHO transfected with wild type α2. Thus, EV1 prefers an inactive integrin α2 for binding. The figure was prepared by modifying figures 2 and 3 of study I.

4.1.2. Under flow conditions, the pre-activation of integrin α2β1 does not increase its binding to collagen I (Study II)

The binding of the CHO- α2E336A and CHO-α2E318W transfectants to collagen was tested under flow conditions with the CELLix instrument. Under static conditions, pre-activation of the receptor (E318W) increases cell spreading slightly, but the inactive mutant (E336A) shows only residual binding to collagen I (Fig. 4A). Under a flow, the situation seems to be completely different. Pre-activation of the receptor does not lead to stronger adhesion to collagen I under the flow, but the non-activated mutant CHO-cells (α2E336A) adhere equally well to collagen I. (Fig. 4B). To study this further, integrin α2β1 was inhibited pharmacologically (Fig. 5 and 6). The development of integrin α2 therapeutics in the collaboration of Professor Jyrki Heino's project and BiotieTherapies had produced some potential small molecule inhibitors for integrin α2. Characterization of the inhibitors revealed that some of them are conformation selective. BTT-3033 blocked the adhesion of CHO- α2E336A significantly better than

the adhesion of α2E318W to collagen 1. In contrast, BTT-3034 blocked the adhesion of the mutants equally well (Fig. 5). BTT-3033 was an even more selective inhibitor for the non-activated conformation under flow (see study II).

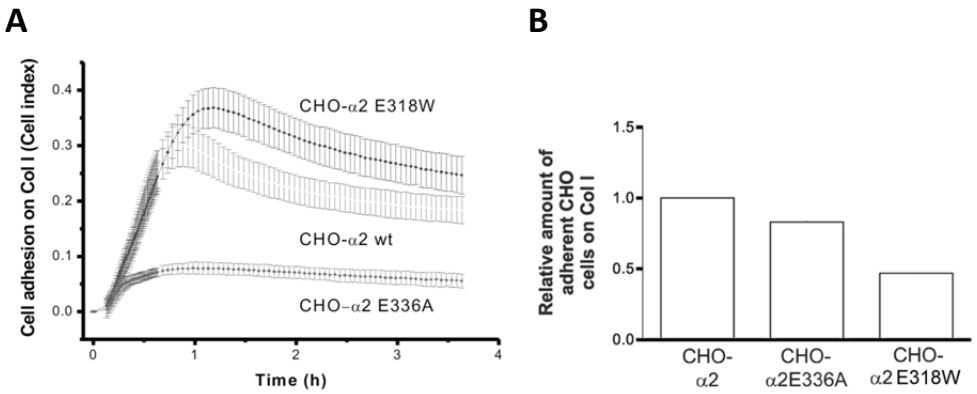


Figure 4. Under flow, integrin α2β1 binds to collagen I without pre-activation. A) The spreading of CHO- α2-transfectants (E336A = non-activated, E318W = pre-activated) under static conditions measured with xCELLigence. B) The binding of same CHO transfectants under flow (90 dynes / cm^2) measured with Cellix. The figure was prepared by modifying figure 5 and supplementary figure 3 of study II.

When the inhibitors were tested with human whole blood, it was discovered that BTT-3033, the inhibitor selective for the non-activated integrin, was effective in inhibiting the collagen 1 induced thrombus formation by platelets (Fig. 6A), whereas the non-selective BTT-3034 inhibited this poorly. The experiment was done with Cellix under flow conditions (Fig. 6B). Thus, it seems that integrin α2β1 on the surface of human platelets binds to collagen I via the non-activated conformation and that pre-activation does not increase its affinity under shear stress. Under static conditions binding via the the non-activated conformation is, in turn, weak.

Leukocyte integrins have been shown to require pre-activation by inside-out signaling for full affinity. Given the fact that their ligands are abundant in the blood circulation and vascular endothelium, this kind of regulatory mechanism seems obvious (Arnaout et al. 2005, Luo et al. 2007). However, α2β1 might not be regulated in this way, since the disruption of the vascular endothelium by trauma is needed to expose the subendothelial collagen to platelets (Sarratt et al. 2005). Another interesting finding was that shear stress seems to increase the binding of non-activated α2β1. A similar phenomenon is seen in in bacterial adhesins. For example, the binding of the FimH adhesin in *E.coli* fimbria is increased 10-fold under shear stress. This can be explained by structural modulation of the protein (Thomas et al. 2002).

Blocking integrin α2β1 is a promising treatment for thrombosis (Nissinen et al. 2010). Our results showed that the conformation of the integrin should be taken into account when designing small molecule inhibitors for integrins.

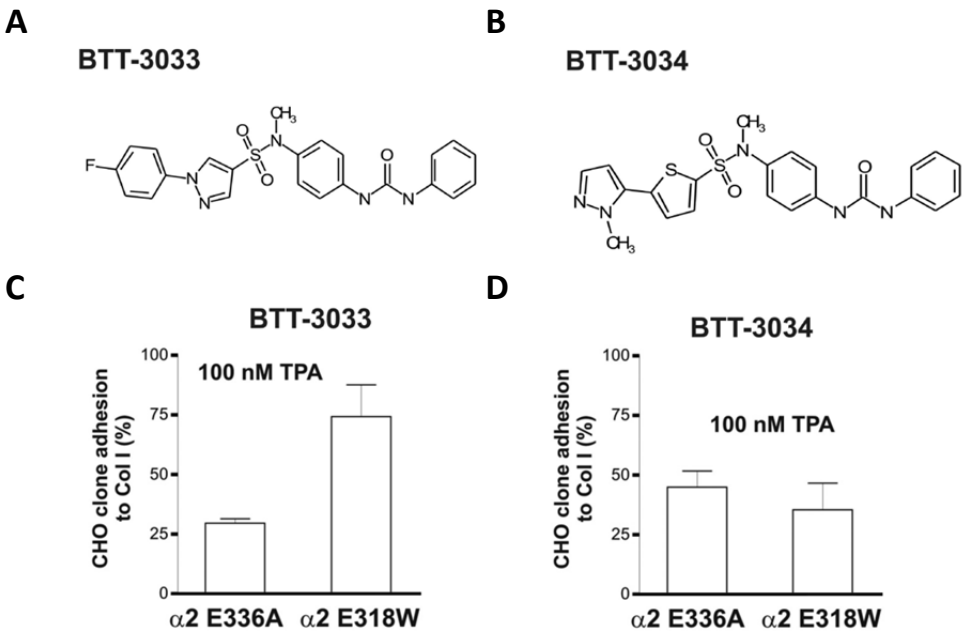


Figure 5. BTT-3033 is selective for the non-activated conformation. A-B) The chemical structure of BTT-3033 and BT-3034 inhibitors. C) The adhesion of CHO-α2-transfectants (E336A = non-activated, E318W = pre-activated) in the presence of the inhibitor BT-3033. D) The adhesion of CHO-α2-transfectants (E336A = non-activated, E318W = pre-activated) in the presence of the inhibitor BT-3034. The assay is a plate and wash –assay under static conditions. 100 % is the adhesion of the cell transfectant without the inhibitor. TPA (12-O-tetradecanoylphorbol-13-acetate) induces the ligand-independent aggregation of integrins and enhances adhesion (Connors et al. 2007). The figure was prepared by modifying figures 1 and 4 of study II.

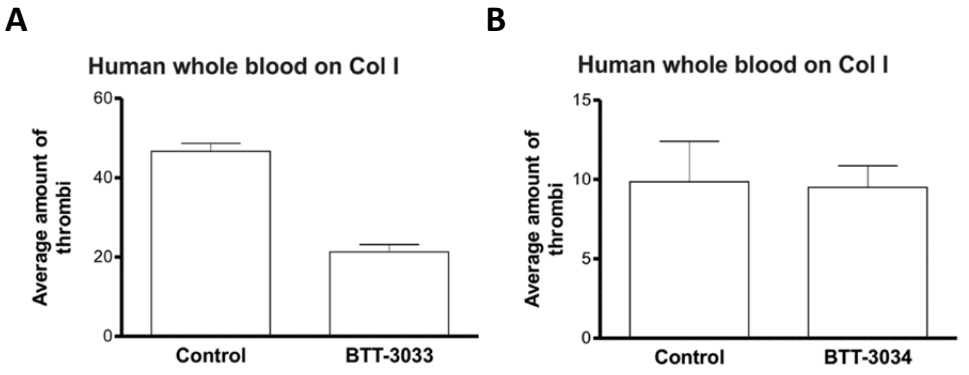


Figure 6. Platelets adhered to collagen I via the non-activated integrin α2β1 conformation under flow for the formation of a thrombus. The collagen induced thrombus formation by platelets was measured under a flow with human whole blood using the Cellix instrument. The inhibition of the formation of a platelet thrombus was more effective by A) the inhibitor BTT-3033 than by B) the inhibitor BTT-3034. BTT-3033 is selective for the non-activated conformation of integrin α2. The figure was prepared by modifying figure 2 of study II.

4.2. Low affinity interaction between HRG and integrin α2β1 inhibits the adhesion of endothelial cells to collagen (Study III)

Histidine-rich glycoprotein (HRG) is a common plasma protein whose concentration in the blood is about 150 µg/ml (Jones et al. 2004). It can affect angiogenesis through the polarization of macrophages and and the normalization of vessels (Rolny et al. 2011). In addition, HRG fragments seems to prevent the motility of endothelial cells (Dixelius et al. 2006). HRG has shown to be a heparin sulfate binding protein. Therefore syndecans, which also have functions related to angiogenesis, could be involved in the process of vessel formation (Vanwildemeersch et al. 2006). Because it is not clear, if HRG has a direct effect on endothelial cells, we decided to study how HRG affects the adhesion of HUVECs (human umbilical vein endothelial cells).

We found that the adhesion of HUVEC cells to collagen I can be blocked by a HRG-treatment. Interestingly, adhesion to vitronectin, fibronectin, or laminin cannot be prevented by HRG (Fig. 7). Thus, there seems to be a specific interaction between HRG and cells.

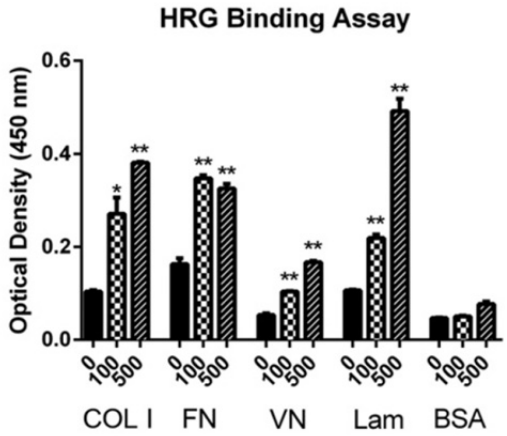


Figure 7. Pre-treatment of HUVECs with HRG inhibits the adhesion of cells to collagen 1, but not to other ECM proteins in a plate and wash -assay. The figure was prepared by modifying figure 1 of study III.

The reason for the inhibition of adhesion can be related to five different hypothetical mechanisms that are listed below:

1) HRG could bind to proteins in the extracellular matrix and mask the ligand binding site of the integrins or other collagen receptors on the cell surface.

2) HRG could bind to an unknown cell surface receptor and transmit a signal, which could affect the inside-out activation of integrins as well as the expression of integrin or syndecan on the cell surface.

3) HRG binds to the collagen receptor integrin complex on the cell surface and inactivates the integrins.

4) HRG can bind to syndecans on the cell surface and prevent their binding to collagen by masking the ligand binding site (glycosaminoglycans).

5) HRG can bind to the collagen receptor integrin and prevent binding to collagen by masking the integrin ligand binding site.

To test the first hypothesis, the binding of HRG to the extracellular matrix was studied with a solid-phase binding assay. In fact, HRG could bind to all of the ECM proteins that were tested. The binding was apparently concentration dependent and no clear differences could be seen between collagen and other proteins (see study III). However, this does not prove that hypothesis number 1 is wrong, because it is possible that in the case of collagen, HRG can mask the ligand binding site of integrin. To exclude hypotheses 1 and 2, we decided to wash the cells after the HRG treatment. If hypothesis 1 were true, a pre-treatment of the cells would not be needed. In contrast, if hypothesis 2 described the mechanism correctly, washing HRG away from the cell surface after a pre-treatment should not have any effect, because the integrin would have already transmitted the signal. The result showed that the pre-treatment of the cells with HRG, before plating, was needed to produce the blocking effect (see study III). In addition, washing the cells after the HRG treatment inhibited the blocking effect of HRG (Fig. 8). Thus, it seems that the blocking mechanism is related to a low affinity interaction between the cell surface and HRG, and it is unlikely to cause by mechnisms postulated by hypotheses 1 or 2. The gene expression profile of the HUVECs was also only minimally altered after the HRG-treatment, suggesting that the binding of HRG to the cell surface does not trigger changes in gene expression (see study III).

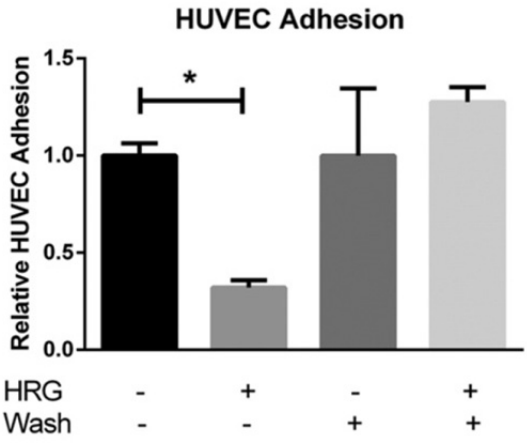


Figure 8. Washing of HUVECs pre-treated with HRG,prevented the HRG-mediated blocking of cell adhesion to collagen 1 in the plate and wash -assay. Thus, hypotheses 1 and 2 are unlikely to be true. The figure was prepared by modifying figure 3 of study III.

To exclude hypothesis 3, we looked at the amount of active β1 integrin on the cell surface with a flow cytometric analysis using an antibody (12G10) that recognizes only

the active β1 integrin. The HRG-treatment does not affect to the amount active β1 integrins (Figure 9). For this reason, it is unlikely that hypothesis 3 is true. Next, we wanted to demonstrate the possible role of syndecans (hypothesis 4) in the HRG-mediated blocking

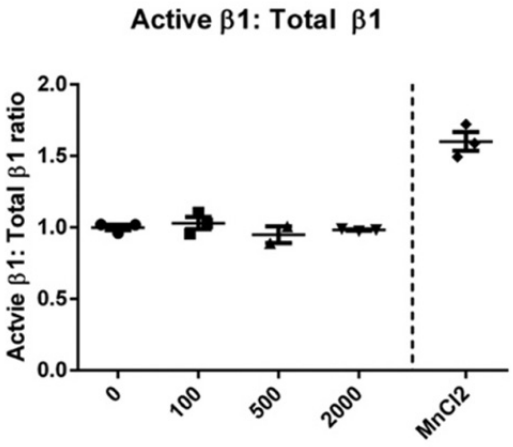


Figure 9. HRG treatment does not change the level of active integrins on the surface of HUVECs so hypothesis 3 is unlikely. The amount of active β1 integrins on the cell surface was measured flow cytometrically with an antibody that recognizes the active conformation of the β1 integrin. The analysis was performed also with an antibody recognizing the total amount of β1 integrins. Mn^{2+} is used as a control because it is known to activate the integrin. The figure was prepared by modifying figure 5 of study III.

mechanism. The adhesion to the collagen mimetic GFOGER peptide seems to be independent of syndecans because the enzymatic removal of cell heparin sulfates on the cell surface (functional components of syndecans) does not affect the spreading of cells on GFOGER (Fig. 10) like it does when cells spread on collagen. Thus, if HRG cannot block the adhesion of cell to GFOGER, the mechanism would be dependent on syndecans. However, the results showed that HRG is able to prevent the adhesion of HUVECs to the GFOGER, suggesting that hypothesis 4 does not describe the mechanism (Fig. 10B).

Based on all our data, the most likely mechanism for the blocking function of HRG is a direct interaction between the αI domain and HRG. Integrin α2β1 is the major collagen receptor integrin expressed by HUVECs (see study III). We could demonstrate with a solid-phase binding assay that this interaction exists. Activation of the integrin (mutation E318W in the α2I domain) increased binding (Fig. 11). Thus, hypothesis 5 seems to be correct. However, the interaction is weak because it could not block the binding of recombinant α2I to collagen in a competition assay (see study III). The assay, in which washing the cells after the HRG pre-treatment inhibits HRG's ability to block the adhesion (Fig. 8), also supports the idea that the interaction is weak.

The typical integrin-ligand interaction is strong (Kd about 100 nM; Käpylä et al. 2000). A biologically relevant low affinity interaction could still take place. Recently, it has been shown that weak receptor-ligand interactions are biologically important but difficult to study. For example, the interaction between an egg cell and a sperm cell is mediated by weak interaction between Juno-Itzumo receptors (Bianchi et al. 2014). Another example is the invasion of the malaria parasite *Plasmodium falciparum* into

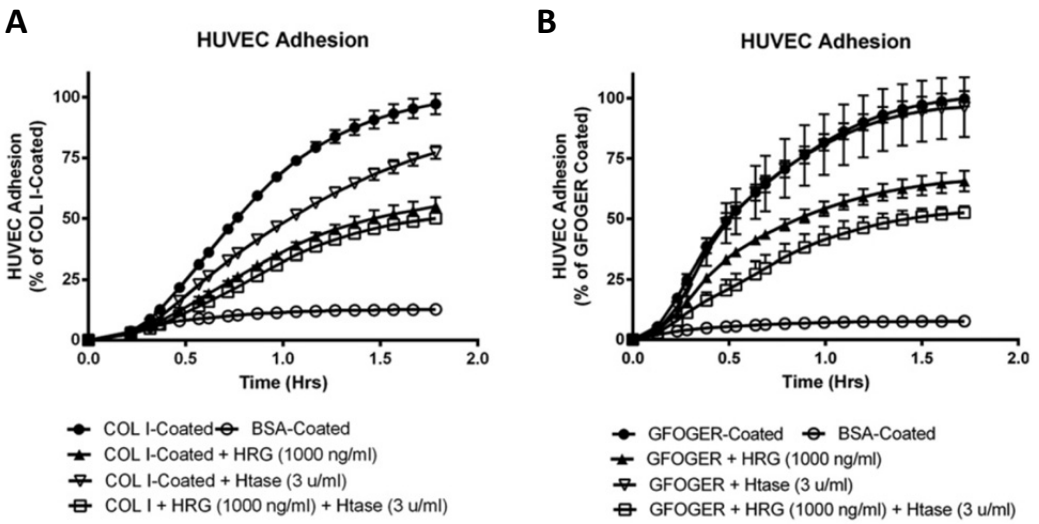


Figure 10. Cell adhesion to GFOGER is independent of heparin sulfate, but HRG inhibits the adhesion of HUVECs also to GFOGER so hypothesis 4 is unlikely. A) Heparitinase treatment (Htase) inhibits the adhesion of cells to collagen 1 but (B) not to the collagen mimetic triple-helical GFOGER peptide. Thus, the adhesion of cells to GFOGER seems to be independent of syndecan. However, HRG can inhibit the adhesion to GFOGER, which indicates that syndecans are not involved in the inhibition of adhesion mediated by HRG. Cell adhesion was measured with an xCELLigence instrument. The figure was prepared by modifying figure 4 study III.

erythrocytes, which is mediated by the weakly interacting PfRh5 and basigin (Crosnier et al. 2011). Given the fact that the concentration of HRG is high in biological fluids (Jones et al. 2004), a low affinity interaction would be an important feature. Many integrins have a role in angiogenesis (Barczyk et al. 2010) and drugs that block integrins have been developed for the suppression of tumour angiogenesis (Mita et al. 2011). Collagen and HRG localize to the same structures in an experimental tumour angiogenesis model so it is possible that the ability of HRG to block the adhesion and migration of endothelial cells is an important mechanism *in vivo* (see study III).

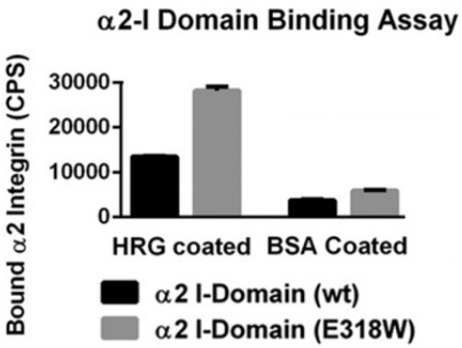


Figure 11. HRG binds to the α2I domain. The integrin α2I domain was able to bind to HRG more than to an unspecific background (BSA) in a solid phase binding assay. The activating mutation E318W increased the binding of the α2I domain to HRG. Thus, hypothesis 5 likely describes the mechanism for the adhesion inhibition. The figure was prepared by modifying figure 6 of study III.

4.3. Extracellular citrullination modulates integrin function (Studies IV-VI)

4.3.1. Cell adhesion can be modulated by extracellular citrullination (Study IV)

The citrullination of collagen II can be detected in the synovium of arthritis patients (Uysal et al. 2009). To study if citrullination affects cell adhesion, we citrullinated collagen II enzymatically (with PAD2 or PAD4) *in vitro* and synthesized collagen mimetic peptides including the citrullinated integrin binding site (GFOGECit). The adhesion of different cell lines to the citrullinated substrate was tested with the xCELLigence instrument. Citrullination seemed to decrease the adhesion and spreading of several cell lines including primary fibroblast-like synoviocytes, mesenchymal stem cells as well as the osteosarcoma cell line Saos-2 (Fig. 12.) The modification does not affect the triple helical structure of the integrin binding site (see study IV).

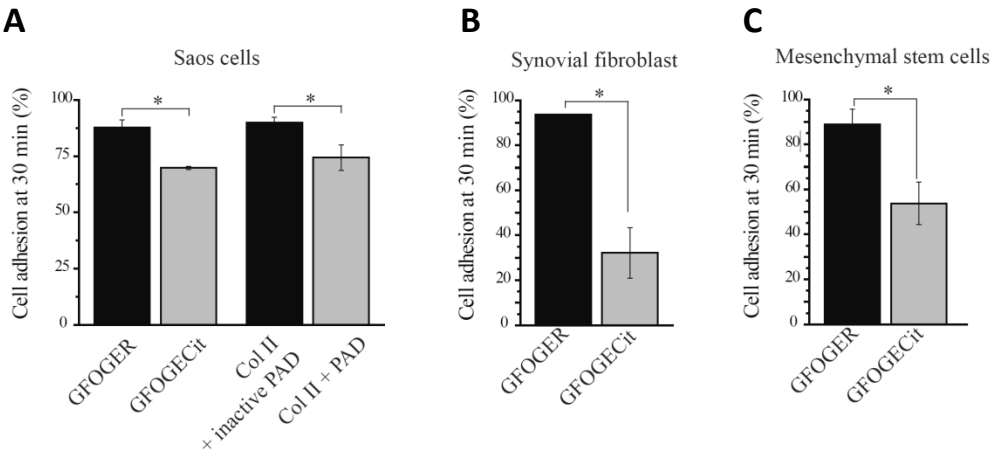


Figure 12. Citrullination of collagen II (CII) and the GFOGER motif decreases cell adhesion. The adhesion of A) the osteosarcoma cell line Saos-2, B) fibroblast like synoviocytes, and C) mesenchymal stem cells to either triple-helical collagen mimetic GFOGER/GFOGECcit peptides or PAD-treated CII (enzymatic citrullination) was measured by xCELLigence technology. The figure was prepared by modifying figures 1 and 7 of study IV.

Interestingly, citrullination blocked the adhesion of synovial like synoviocytes and mesenchymal stem cells more effectively than Saos-2 cells (Fig. 12). The most straightforward explanation for the result is that the cell lines use different collagen receptor integrins for binding to collagen. Saos-2 cells express integrin α1β1 and α11β1 (Mirtti et al. 2006). Synovial fibroblasts express at least α1β1 (Pirilä et al. 1996), but also likely α11β1 (Tiger et al. 2001). The binding of mesenchymal stem cells to collagen is critically dependent on α2β1 and α11β1 (Popov et al. 2011). To test this

hypothesis, we used CHO cells transfected with integrin α1, α2, α10, and α11 to measure how specific integrins recognize the citrullinated form of the integrin binding site in collagen. CHO cells do not expresss collagen receptors on their surface (Käpylä et al. 2004). Our results showed that citrullination has a minor effect or no effect at all on the integrin α1β1 and α2β1 mediated cell adhesion but in the case of α10β1 and α11β1 integrin, cell adhesion was strongly decreased (Fig. 13). The results were confirmed also with recombinant integrin αI-domains in a solid-phase binding assay. Molecular modeling was used to explain differences in integrin binding (see study IV).

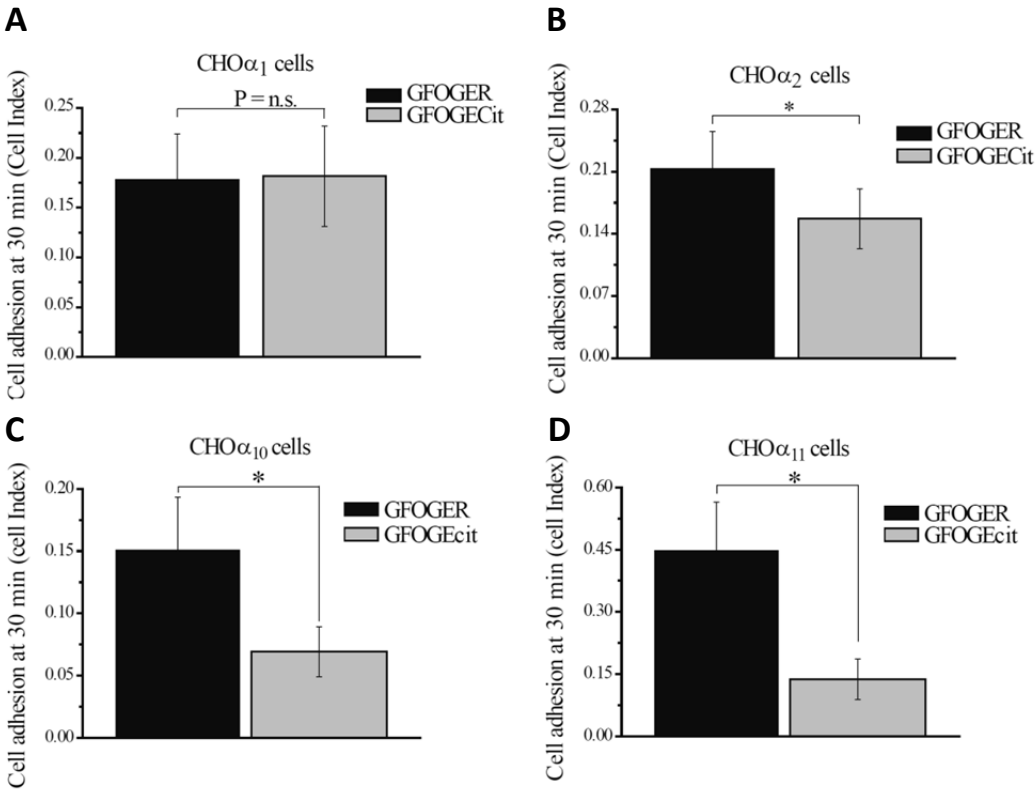


Figure 13. Citrullination of the integrin binding site GFOGER only slightly affects α1β1 and α2β1 mediated cell adhesion but dramatically decreases C) α10β1 and D) α11β1 mediated cell adhesion. The adhesion of CHO-integrin transfectants was measured with xCELLigence technology. The figure was prepared by modifying figures 2-5 of study IV.

Collagen receptor integrins regulate the modeling of the extracellular matrix by regulating the expression of genes coding for matrix metalloproteinases and collagen (Riikonen et al. 1995). In addition they can be important for cell growth (Popova et al. 2007). Because the survival of mesenchymal stem cells seems to be critically linked to the cell adhesion mediated by integrin α11β1 (Popov et al. 2011), we tested how mesenchymal stem cells can survive in serum-free medium when adhered to GFOGECit. Our results showed that although mesenchymal stem cells attached to GFOGECit, they detached and clumped together more quickly when plated on GFOGERCit than GFOGER (Fig. 14). The citrullination of collagen seems to

dramatically affect the integrin-mediated adhesion of synovial-like fibroblast and mesenchymal stem cells (Fig. 12A-B). Both cell types are present in the synovium (MacFarlane et al. 2013). Mesenchymal stem cells are interesting in the context of rheumatoid arthritis, because they are immunosuppressive and have been shown to participate in the pathological processes of arthritis (Marinova-Mutafchieva et al. 2002).

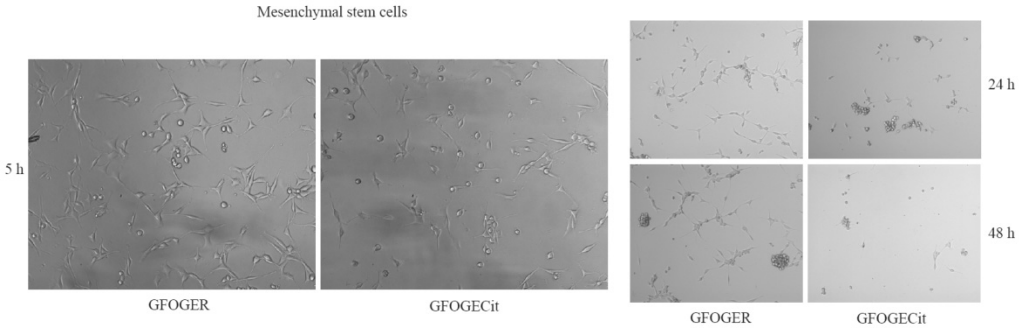


Figure 14. Citrullination of the integrin binding motif GFOGER decreases the survival of mesenchymal cells. Mesenchymal stem cells were plated on GFOGER or GFOGEcit in serum free medium. The cells adhered to both peptides (5 h), but started to detach and clump more quickly in the case of GFOGEcit (24 h and 48 h). The figure was prepared by modifying figure 7 of study IV.

We have not characterized the frequency of citrullination in GFOGER-type integrin binding sites of collagen II *in vivo*, which makes it difficult to estimate the effect of the citrullination of the integrin binding site in a physiological situation. The important role of collagen receptor integrins in processes relevant to arthritis has been shown in different animal models. The pharmacological inhibition of α1β1 and α2β1 ameliorates experimental arthritis (De Fougerolles et al. 2000) and an α2 deficiency also decreases the amount of cartilage destruction (Peters et al. 2012). However, citrullination does not affect the binding of integrins α1β1 and α2β1. Thus, it is implausible that the beneficial effects of the citrullination of collagen II are mediated by these integrins. On the other hand, integrin α10β1 is expressed in the cartilage and in chondrocytes and the consequence of the α10β1 knockout is damage to the joints at least in dogs (Kyöstilä et al. 2013). Altogether, inhibiting α10β1 and α11β1 mediated adhesion to collagen by citrullination may have a role in the pathogenic destruction of joints in arthritis.

4.3.2. The function of extracellular matrix associated growth factors can be modulated by citrullination (Study V)

Besides cell adhesion, the ECM also regulates cell behavior by acting as a depository for growth factors (Hynes 2009). Because of this, extracellular citrullination may potentially affect the function of growth factors associated with the extracellular matrix. To further examine this hypothesis, we decided to focus on transforming

growth factor beta (TGF-β), which is a physiologically and pathologically important growth factor (Margadant and Sonnenberg 2010). TGF-β is bound to the extracellular matrix in its latent form and it becomes activated by the binding of αV integrins to the RGD of LAP (Munger et al. 1999).

To test if the function of ECM associated TGF-β can be modulated by extracellular citrullination, we produced ECMs with CHO-LTBP1 transfectants (Unsöld et al. 2001). The treatment of the ECMs with the PAD2 enzyme led to a decrease in TGF-β signaling measured by the co-culture of the MLEC-PAI-1/Lu reporter cells and HaCaTs (Fig. 15). The reason for this can be either the inhibition of integrin mediated activation or the inhibition of the active TGF-β binding to its receptor.

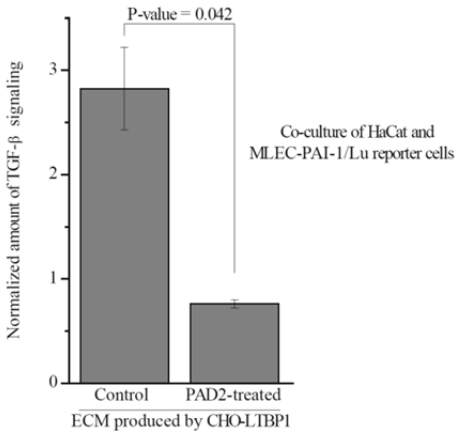


Figure 15. PAD-treatment inhibits the function of ECM associated TGF-β. The ECM of CHO-LTBP1 cells was treated with the PAD enzyme. After the treatment, the amount of TGF-β activated by HaCaT cells was measured by the MLEC-PAI-1/Lu reporter cell assay. The signal was normalized to the signal of 0.5 ng/ml TGF- β in the reporter assay. The figure was prepared by modifying figure 2 of study V.

To answer the question, how citrullination affects the integrin mediated activation of TGF-β, we treated the recombinant TGF-β1-latency associated peptide (β1-LAP) with PAD2 or PAD4 and measured how integrins bind after citrullination. Our results showed that the binding of the recombinant integrin αVβ6 ectodomain is decreased due to the citrullination of the RGD in β1-LAP (Fig. 16A). The active TGF-β1 was also citrullinated and its binding to the TGF-RII was assayed. The results showed that citrullination decreases the binding of active TGF- β1 to its receptor (16D). Thus, extracellular citrullination can prevent TGF-β signaling by inhibiting the integrin mediated activation of the ECM associated TGF-β and by preventing the binding of TGF- β1 to its receptor. In both cases the structural explanation for the prevention was the loss of strong ionic interactions (Fig. 16 B-C and E-F).

TGF-β is a well-known regulator of the immune system (Yoshimura et al. 2010). The loss of TGF-β1 or its integrin mediated activation leads to severe multiorgan inflammation (Yang et al. 2007). The major role of TGF-β signaling in immune cells is to induce tolerance but, it has opposite functions too. TGF-β has pleiotropic functions in rheumatoid arthritis. The systemic blocking of TGF-β activity worsened the joint inflammation (Wahl et al. 1993), but local inhibition in the joints suppresses the experimental arthritis (Thorbecke et al. 1992). Thus, the modulation of ECM associated growth factors, like TGF-β, via citrullination is a potential pathological

mechanism behind the progression of rheumatoid arthritis. In addition, citrullination seems to be linked to other auto-inflammatory diseases (György et al. 2006).

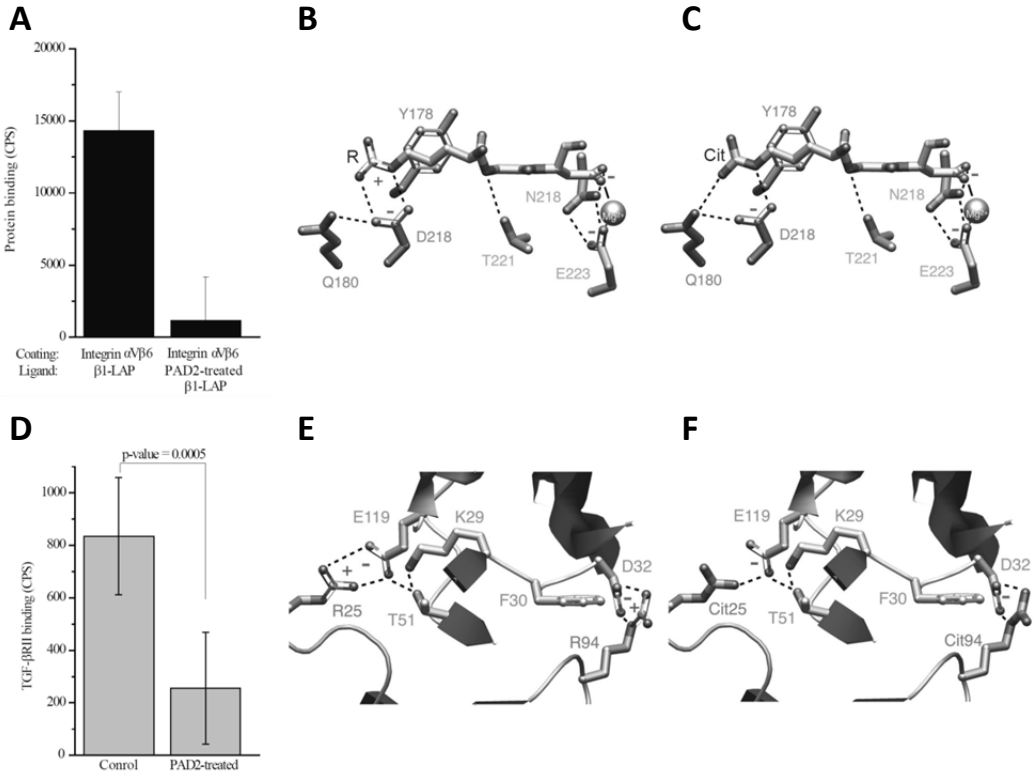


Figure 16. Citrullination inhibits the function of TGF-β by two different mechanisms. A) The treatment of recombinant TGF-β-LAP by the PAD enzyme leads to the citrullination of RGD in LAP. This prevents the binding of integrin αVβ6, which is an important step in the activation of latent TGF-β. B-C) The structural explanation for the effect of citrullination on the binding of integrin to LAP is the loss of the strong ionic bond between the arginine in the RGD of LAP and D218 in αVβ6. D) In addition, the enzymatic citrullination of active TGF-β inhibits its binding to the TGF-β receptor II (TGF-βRII), which is needed for the induction of TGF-β signaling. E-F) In this case, citrullination breaks the strong ionic bond between R25 in active TGF-β and E119 in TGF-βRII. The figure was prepared by modifying figures 2 and 6 of study V.

4.3.3. Integrin binding sites become citrullinated in joint inflammation in vivo (Study VI)

The citrullination of extracellular proteins and extracellular PAD activity have been reported in joint inflammation (van Beers et al. 2013, Damgaard et al. 2014). But, the occurrence of extracellular PAD activity and the targets of citrullination *in vivo* have been poorly characterized. Although the functional consequences of citrullination on extracellular proteins have been suggested (Okumura et al. 2009, Shelef et al. 2014), an analysis of the citrullination of the functional arginine residue *in vivo* is lacking. To find out if the integrin binding sites or other functional arginine residues are

citrullinated *in vivo*, we analyzed about 40 synovial fluids from patients suffering from seropositive and negative rheumatoid arthritis as well as from patients with juvenile idiopathic arthritis, psoriatic arthritis, gout, and ankylosing spondylitis. A PAD activity assay and mass spectrometry were used in the analyses.

The results showed that citrullination is not specific for any joint disease, but seems to be a general inflammation dependent phenomenon (Fig. 17). The presence of citrullinated proteins correlated positively with the number of infiltrated leukocytes (Fig. 17C) in the joint, and citrullination was not detected in synovial fluids that were considered non-inflammatory (white blood count less than 200E6 /dm^3) (Fig. 17A). Interestingly, no differences can be seen between cases of rheumatoid arthritis positive and negative for the anti-citrullinated protein antibody (ACPA) (Fig. 17B), indicating that citrullination alone cannot trigger the ACPA response.

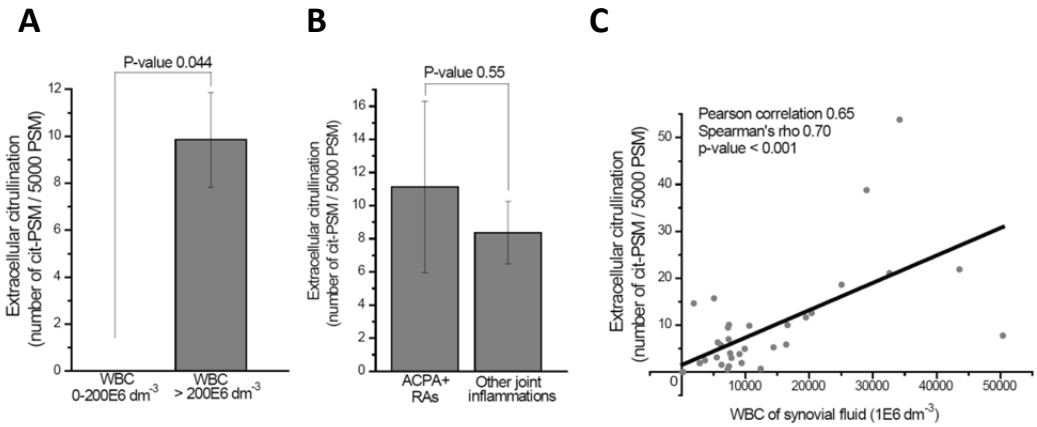


Figure 17. Extracellular citrullination is an inflammation dependent process. A) Citrullination cannot be identified in synovial fluids in which the amount of leukocytes (white blood count, WBC) is lower than 200E6 dm^{-3}. Clinically these synovial fluids are considered to be non-inflammatory. B) The synovial fluids from rheumatoid arthritis patients (RAs) positive for the anti-citrullinated protein antibody (ACPA+) do not show increased citrullination compared to other patients with joint inflammation due to other causes but C) the presence of citrullinated extracellular proteins correlated with leukocyte infiltration to the joints. The analysis was performed by mass spectrometry and the amont of citrullination in extracellular proteins was normalized to the number of all peptide spectrum matches (PSM) of the sample. The figure was prepared by modifying figure 2 of study VI.

Based on the identification of heparin-agarose bound proteins by mass spectrometry, the citrullination of 24 different extracellular proteins can be detected (Fig. 18) *in vivo*. Many proteins have more than one arginine residues that becomes citrullinated (see study VI). The citrullination of most extracellular proteins was identified in under 15 % of the samples but angiogenin, fibrinogen, fibronectin and proteoglycan 4 were citrullinated in more than 40 % of the samples. The extracellular matrix proteins whose citrullination could be detected *in vivo* were collagen III, cartilage oligomeric matrix protein, EGF-containing fibulin-like extracellular protein, fibrillin-1, fibrinogen,

fibronectin, matrix-remodelling-associated protein 5, papilin, pro-collagen C-endopeptidase enhancer 1, and proteoglycan 4 (Fig. 18A).

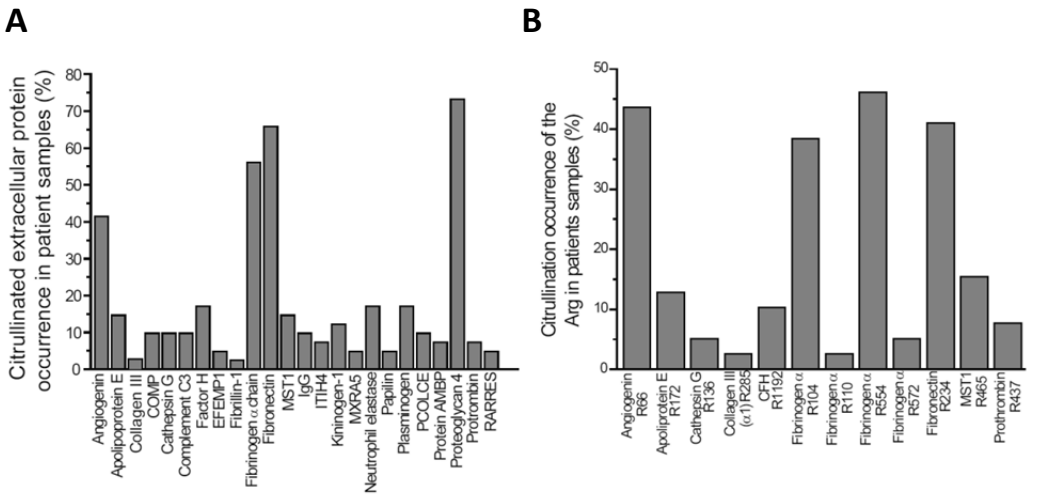


Figure 18. A) The most common citrullinated extracellular proteins and B) the citrullinated functional arginine residues *in vivo* based on a mass spectrometric analysis of synovial fluids. Interestingly, integrin binding sites in collagen III (R285 of GAOGER motif), in fibronectin (R234 of isoDGR motif) and in fibrinogen (R572 of RGD motif) are among the *in vivo* targets of the PAD enzymes. The figure was prepared by modifying figures 1 and 5 of study VI.

Interestingly, over 20 % of the arginines that become citrullinated have characterized functions *in vivo* (see the study VI). The most commonly found citrullinated arginine residues were R66 in angiogenin, R104 and R554 in fibrinogen as well as R234 in fibronectin (Fig. 18B). R66 is needed for the angiogenic activity of angiogenin (Shapiro and Vallee (1992). R104 is in the plasmin cleavage site of fibrinogen (UniProtKB - P02671 (FIBA_HUMAN)) and R554 is needed for the polymerization of fibrin (Benson et al. 1993, Koopman et al. 1993). R234 is an integrin binding site in fibronectin (Curnis et al. 2006). Other integrin binding sites that can be citrullinated *in vivo* are R572 in fibrinogen (RGD) (Plow et al. 1987) and R285 in collagen (GAOGER) (Siljander et al. 2004). Thus, the citrullination of functional arginine residues, including the integrin binding motifs NGR/isoDGR, RGD, and GAOGER, can be the targets for citrullination *in vivo*, which confirms the earlier suggestion that the posttranslational modification of integrin binding motifs can be a pathological mechanism contributing to diseases.

Although the citrullination of arginines can be detected *in vivo*, it is not clear if the citrullination of the arginine has any functional consequences. R234 in fibronectin was selected for a further analysis. R234 belongs to the binding site of αVβ3 integrin NGR/isoDGR in the fifth of type 1 fibronectin domain. The asparagine 232 can undergo spontaneous conversion to isoaspartate, which seems to increase integrin binding (Curnis et al. 2006). The *in vivo* role of the NGR/isoDGR site is not known, but it can have a role in fibronectin fibrillogenesis (Takahashi et al. 2007).

In order to study the binding of integrin αVβ3 to citrullinated isoDGR, we measured the binding of the recombinant integrin ectodomain to the N-terminal 30 kDa fragment of fibronectin. The results suggested that citrullination prevents the binding of integrin αVβ3 to the citrullinated isoDGR (Fig. 19). It is possible that the citrullination of other arginine residues in the N-terminal 30 kDa fragment of fibronectin also affect integrin binding, but the NGR/isoDGR site is the only known integrin binding motif in that region (Leiss et al. 2008). Citrullination eliminates the strong electrostatic interaction between the arginine residue of isoDGR and aspartate 150 in the integrin αV subunit. This likely explains why citrullination inhibits the interaction. Residual binding can still take place due to hydrogen bonding. However, hydrogen bonds are weak compared to strong ionic bonds and are unlikely able to rescue the changes (Fig. 19A-B).

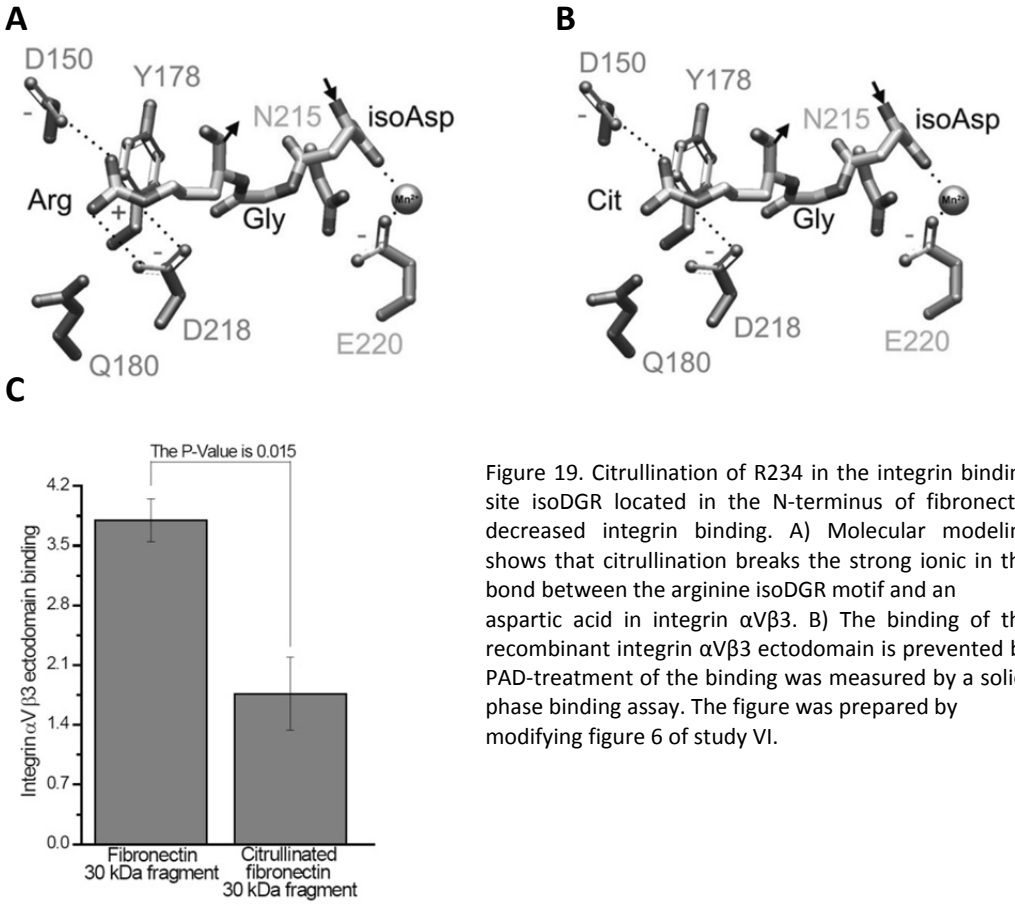


Figure 19. Citrullination of R234 in the integrin binding site isoDGR located in the N-terminus of fibronectin decreased integrin binding. A) Molecular modeling shows that citrullination breaks the strong ionic in the bond between the arginine isoDGR motif and an aspartic acid in integrin αVβ3. B) The binding of the recombinant integrin αVβ3 ectodomain is prevented by PAD-treatment of the binding was measured by a solid-phase binding assay. The figure was prepared by modifying figure 6 of study VI.

To study PAD selectivity *in vivo*, we analyzed the citrullination sites in the known structures of the proteins in the Protein Data Bank (PDB). This *in silico* analysis showed that nearly all of the identified citrullinated arginine residues are exposed (Fig. 20A). Given the fact that many functional arginines need to be exposed for normal function, such as receptor binding (see study V), they are likely to be optimal

substrates for the PAD enzyme and are frequently citrullinated *in vivo.* The arginines that were buried inside the protein should be part of conformational changes that reveals the arginine residue for PAD. A potential process can be proteolysis that is very active in the synovium in rheumatoid arthritis (Okada et al. 1986). Because citrullination has been suggested to be a more or less selective process, the amino acid in the N-2 position from the citrullinated arginine as well as the loop structure have been thought to mediate selectivity (Arita et al. 2006). However, our data showed that the location of the arginine in the secondary structure and the amino acid in the N-2 position can be anything (20B-C).

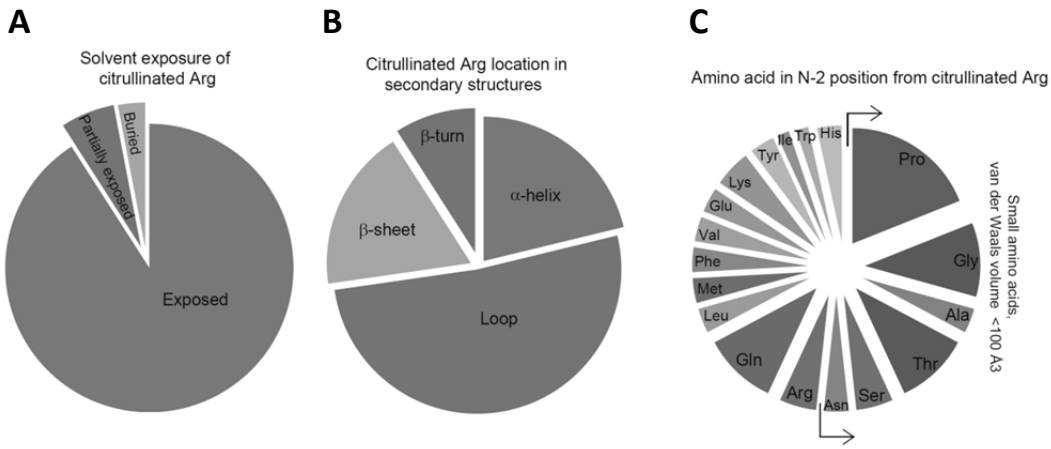


Figure 20. The structural features related the citrullination sites *in vivo*. A) Solvent exposure, B) secondary structure localization, and C) N-2 amino acid residue of the arginine residues that are targets for citrullination *in vivo*. The analysis is based on the existing protein structures in PDB (Protein Data Bank). Based on this analysis, the exposure of the arginine residue to solvent is the only obvious structural characteristic that dictates the selectivity of extracellular PAD enzymes in joints *in vivo*. The figure was prepared by modifying figure 3 of study VI.

Thus, citrullination seems to be a relatively unselective process and basically any exposed arginine can be a target for extracellular citrullination. Due to the structure of the protein complex in a biological context, the PAD enzymes may favour some arginine residues, while steric hindrance may prevent interactions with others. This might explain the *in vivo* selectivity at least partly. It is also known that some regulatory proteins can bind to PAD enzyme *in vivo* (Chang et al. 2015).

5. CONCLUSIONS

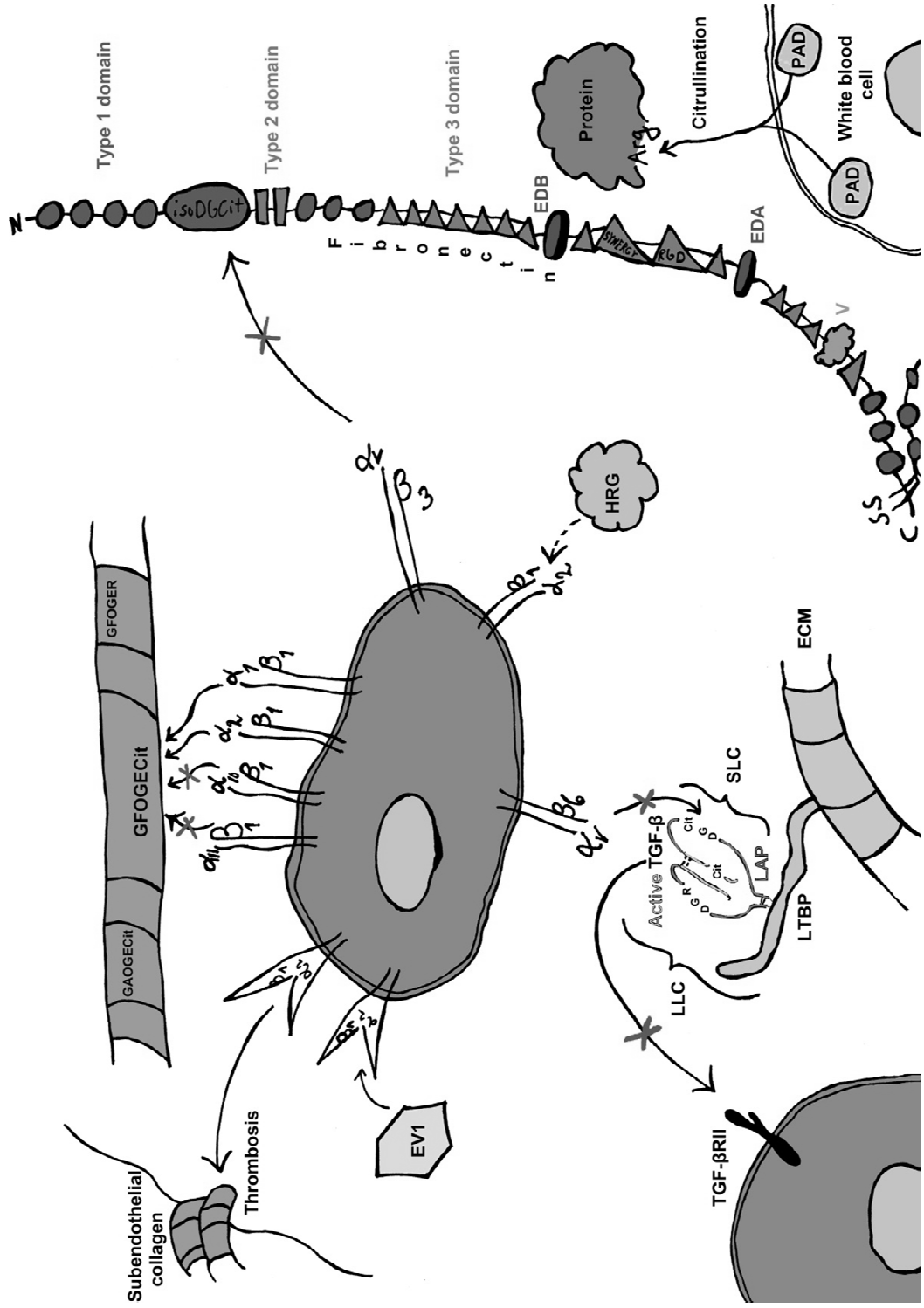
Conventionally, integrins have been thought to bind to large ligands in their pre-activated conformation with high affinity (Luo and Springer 2006, Hynes 2002). However, in this thesis we show that (Fig. 21):

1) integrin α2β1 binds to Echovirus 1 in its non-activated conformation. In addition, the non-activated integrin α2β1 binds to collagen I under flow conditions as efficiently as the pre-activated conformation (studies I and II).

2) the interaction between histidine-rich glycoprotein and integrin α2β1 can be characterized as a low affinity binding event. HRG blocks the binding of integrin α2β1 to collagen I (study III).

3) arginines in the integrin binding sites can be posttranslationally modified by citrullination *in vivo*. Citrullination can modulate the integrin-mediated behavior of cells in a receptor-specific manner (studies IV-VI).

Figure 21 (next page). Unconventional integrin-ligand interactions can be physiologically important; a schematic illustration of the results (see sections 5 and 6). EV1 binds to the non-activated conformation of integrin α2β1. In addition, the non-activated conformation of integrin α2β1 is the primary conformation for the integrin when it binds to collagen under flow. The plasma protein HRG interacts with integrin α2β1 with low affinity, which prevents the integrin α2β1 –mediated adhesion of endothelial cells to collagen. This can contribute to angiogenesis. Integrin ligands can be posttranslationally modified by the extracellular PAD enzyme that is released from activated leukocytes. The citrullination of collagen decreased the binding of integrins α10β1 and α11β1 dramatically, but the binding of other collagen receptors is altered only slightly. Extracellular citrullination can also block the binding of integrin to fibronectin. Besides adhesion, extracellular PAD can inhibit the function of ECM-associated TGF-β. The citrullination of GAOGER (a GFOGER-type low affinity recognition motif of collagen receptors) and isoDGR (the integrin αVβ6 binding motif in the N-terminus of fibronectin) can be found *in vivo* in joint inflammation.


Type 1 domain
Type 2 domain
Type 3 domain
N
isoDGCit
Fibronectin
EDB
EDA
V
C
Protein
Arg.
Citrullination
PAD
PAD
White blood cell
HRG
GFOGER
GFOGECit
GAOGECit
Subendothelial collagen
Thrombosis
EV1
Active TGF-β
LAP
LLC
SLC
LTBP
ECM
TGF-βRII

6. FUTURE PROSPECTS

The essential questions raised by the results of this thesis are listed below.

What is the structure of the focal adhesion complex when Echovirus 1 binds to integrin α2β1?

The structures of focal adhesion complexes have been studied recently by mass spectrometry (Jones et al. 2015). Because EV1 binds to the non-activated conformation of integrin α2β1, it provides a model system for studying the integrin tail-complex in inactive integrins. In addition, the conformational states of integrin and the structure of the virus-integrin complex can be studied by super resolution optical microscopy and electron microscopy.

What is the effect of the conformation selective inhibitor in vivo?

In Study II we showed that the inhibitor BT-3033, which is selective for the non-activated integrin α2β1, blocked the adhesion of platelets to collagen I under flow more effectively than BT-3034, which is not selective for the integrin's conformation. It would be interesting to study, how effective the inhibitors are in different thrombosis models *in vivo*. The effects of the inhibitors on inflammation models *in vivo* have already been published (Nissinen et al. 2015).

How is the PAD enzyme released from the cell?

Based on the data produced by our laboratory and the others (Spengler et al. 2015), the primary cause for the extracellular citrullination is the presence of activated neutrophils, which suggests that the formation of neutrophil extracellular traps is an important mechanism leading to extracellular citrullination. However, other mechanisms can still exist. It is not known if the release of PAD is a side product of inflammatory process or if it also has an immunological role. The extensive citrullination of fibrinogen *in vivo* would suggest that the extracellular citrullination could prevent the clotting of fibrin during inflammation. Given the fact that extracellular citrullination is an inflammation dependent process and citrullinated proteins more immunogenic than their unmodified counterparts (Lundberg et al. 2005), it is possible that citrullination needs to occur outside the cell in order to participate in the immune defense against pathogens.

What regulates PAD selectivity in vivo?

Based on our data, there are no obvious structural features that would dictate the selectivity of PAD. The activity of PAD can be regulated by PTPN22 (Chang et al. 2015) and calcium influx (Romero et al. 2013), but it is likely that PAD is regulated by other

regulatory mechanisms too. Interestingly some *in vivo* PAD ligands, like trichohyalin, are calcium-binding proteins (Vossenaar et al. 2003). This would suggest a model, where PAD activity can be controlled by calcium binding regulatory proteins. Another interesting hypothesis would be an enzyme that could remove the peptidyl citrullination. However, the existence of such an enzyme has not been reported.

What are the effects of PAD-inhibition on integrin mediated processes in vivo?

Although we showed that integrin binding sites can be citrullinated *in vivo*, it is not known if the pathophysiological processes of inflammatory diseases are affected by this phenomenon. *In vivo* studies with pharmacological inhibition of PAD would be needed to answer this question. In addition, the citrullination of extracellular matrix associated growth factors has not yet been shown *in vivo*.

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