Integrin $\alpha V \beta 3$-Directed Contraction by Connective Tissue Cells

Role in Control of Interstitial Fluid Pressure and Modulation by Bacterial Proteins

ÅSA LIDÈN
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Abstract


This thesis aimed at studying mechanisms involved in control of tissue fluid homeostasis during inflammation.

The interstitial fluid pressure (Pᵢ) is of importance for control of tissue fluid balance. A lowering of Pᵢ in vivo will result in a transport of fluid from the circulation into the tissue, leading to edema. Loose connective tissues that surround blood vessels have an intrinsic ability to take up fluid and swell. The connective tissue cells exert a tension on the fibrous network of the tissues, thereby preventing the tissues from swelling. Under normal homeostasis, the interactions between the cells and the fibrous network are mediated by β1 integrins. Connective tissue cells are in this way actively controlling Pᵢ.

Here we show a previously unrecognized function for the integrin αVβ3, namely in the control of Pᵢ. During inflammation the β1 integrin function is disturbed and the connective tissue cells release their tension on the fibrous network resulting in a lowering of Pᵢ. Such a lowering can be restored by platelet-derived growth factor (PDGF) -BB. We demonstrated that PDGF-BB restored Pᵢ through a mechanism that was dependent on integrin αVβ3. This was shown by the inability of PDGF-BB to restore a lowered Pᵢ in the presence of anti-integrin β3 IgG or a peptide inhibitor of integrin αVβ3. PDGF-BB was in addition unable to normalize a lowered Pᵢ in β3 null mice. Furthermore, we demonstrated that extracellular proteins from Streptococcus equi modulated αVβ3-mediated collagen gel contraction. Because of the established concordance between collagen gel contraction in vitro and control of Pᵢ in vivo, a potential role for these proteins in control of tissue fluid homeostasis during inflammation could be assumed. Sepsis and septic shock are severe, and sometimes lethal, conditions. Knowledge of how bacterial components influence Pᵢ and the mechanisms for tissue fluid control during inflammatory reactions is likely to be of clinical importance in treating sepsis and septic shock.

Keywords: interstitial fluid pressure, collagen gel contraction, integrin αVβ3, septic shock, fibronectin

Åsa Lidén, Department of Medical Biochemistry and Microbiology, Box 582, Uppsala University, SE-75123 Uppsala, Sweden

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List of papers

This thesis is based on the following papers, referred to in the text by their roman numerals:


Contents

Introduction ........................................................................................................... 7
Connective tissue and extracellular matrix .................................................... 7
  Collagens ........................................................................................................ 8
Fibronectin ..................................................................................................... 10
Integrins .......................................................................................................... 11
  Integrin families .......................................................................................... 16
Platelet-derived growth factor ...................................................................... 16
Inflammation .................................................................................................... 18
  Sepsis and septic shock ............................................................................... 19
    Gram-negative bacteria in sepsis .............................................................. 19
    Gram-positive bacteria in sepsis ............................................................... 20
Streptococcus equi .......................................................................................... 20
Interstitial fluid pressure ............................................................................... 21
Collagen gel contraction ............................................................................... 23

Present investigations .................................................................................... 25
  Aim ................................................................................................................ 25
Methods .......................................................................................................... 25
  Collagen gel contraction measurements .................................................. 25
    Interstitial fluid pressure measurements ................................................. 26
Results ............................................................................................................. 27
  Paper I .......................................................................................................... 27
  Paper II .................................................................................................... 28
  Paper III ................................................................................................... 29

Discussion and future perspectives ............................................................... 31
  Concluding remarks .................................................................................... 33

Acknowledgements ......................................................................................... 34

References ....................................................................................................... 36
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
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<td>3D</td>
<td>Three dimensional</td>
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<td>COL domain</td>
<td>Collagen domain</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>ERK</td>
<td>Extracellular-regulated kinase</td>
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<td>FA</td>
<td>Focal adhesion</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>FN</td>
<td>Fibronectin</td>
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<td>IFP or P_{IF}</td>
<td>Interstitial fluid pressure</td>
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<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
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<td>LBP</td>
<td>LPS-binding protein</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LTA</td>
<td>Lipoteichoic acid</td>
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<td>NC domain</td>
<td>Non-collagen domain</td>
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<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>P_{IF} or IFP</td>
<td>Interstitial fluid pressure</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
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<td>RGD</td>
<td>Arginine–Glycine–Aspartic acid</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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Introduction

All nucleated cells need to be anchored to an extracellular matrix (ECM) to exert their differentiated functions and to survive [1]. Integrins are a family of major adhesion receptors that mediate contacts between cells and the ECM [2]. Signaling mechanisms triggered by integrins have been studied mainly in cultured cells [3]. Since there presumably are large differences between cellular responses in vitro and in vivo it is difficult to know the relevance of such in vitro findings. This thesis deals with the role of integrins in generating tensile forces, which in turn are of importance for control of interstitial fluid pressure in vivo. We combined studies on cultured cells with animal experiments in mice. This allowed evaluation of the in vivo importance of the findings obtained in in vitro cell culture studies.

Connective tissue and extracellular matrix

The connective tissues support the different organs and structures of the body [4]. They consist of cells embedded in extracellular matrix (ECM) and include bone, cartilage and loose connective tissue. The connective tissue cells are of mesenchymal origin. Osteoblasts, chondrocytes and fibroblasts are example of connective tissue cells. The ECM is secreted by the connective tissue cells and is composed of polysaccharides and a number of structural and adhesive proteins [5]. The composition is dependent on the function of the connective tissue in question. Collagens, fibronectins, laminins, elastin, fibrillin, hyaluronan, versican and aggrecan are examples of ECM components. Collagens are the most abundant protein components of the ECM and create the structural framework of the matrix [6]. Fibronectins and laminins are adhesive components of the ECM and elastin and fibrillins form the elastic fibers [7-10]. The glycosaminoglycan hyaluronan and proteoglycans such as versican and aggrecan are examples of space-filling molecules. These molecules are relatively inflexible and negatively charged. Because of this they are present in an extended form in the matrix, occupying a relatively large volume compared to their molecular mass. The negatively charged molecules attract cations creating an osmotic pressure that causes fluid to be transported into the matrix. The swelling that follows gives the connective tissues their ability to resist compressive forces [11-13].
The loose connective tissue is composed of a ground substance (space-filling molecules), tensile and elastic fibers and glycoproteins as well as cellular components including fibroblasts and nerve endings (Figure 1). The ground substance of the connective tissue, the elastic fibers and the adhesive glycoproteins interact with the collagen framework of the ECM and create supramolecular complexes. The connective tissue cells interact with the collagenous structural framework and together with the interactions with elastic fibers and adhesive proteins they restrain the ground substance (proteoglycans and hyaluronan) and keep the tissue from swelling [5, 14]. The connective tissue cells are in this way actively involved in control of tissue fluid homeostasis, the mechanisms for this will be further discussed below, in the section about interstitial fluid pressure.

The work included in this thesis mainly focus on interactions between cells, collagen and fibronectin and these two ECM proteins are therefore reviewed in further detail.

Collagens

Collagens are the most abundant proteins in the ECM and also in the human body in total. Besides their important function as structural components of the connective tissues, they have a number of other important functions [15]. Cells adhere to collagens via integrins (see below), discoidin domain receptors [16] or glycoprotein VI [17], the latter only expressed on platelets. Cell adhesion to collagens are involved in cell migration, and interactions between collagens and cells regulate tissue remodeling during growth, differentiation and wound healing among other things [15]. Collagen molecules are homo- or heterotrimeric proteins built of three polypeptide α chains [4, 18]. There are at least 27 different collagens in vertebrates formed by 42 distinct α chains [15]. The collagen (COL) domains are triple helical domains formed by a repeating number of Gly-X-Y motifs in the amino acid sequence of each α chain, where X is often proline and Y 4-hydroxyproline. Each of the α chains form a left-handed helix and the three α chains coil together into a right-handed coiled-coil triple helix. The Gly-X-Y motif is crucial for the structure since the glycine residue is the only amino acid small enough to fit into the inner part of the triple helix. The presence of 4-hydroxyproline stabilizes the helix by participating in lateral hydrogen bonds [19]. It is the presence of at least one of these COL domains that defines the members of the collagen superfamily. This family is a diverse family of proteins with members that differ both in molecular organization and tissue distribution and function [15, 20]. In addition to the COL domains, many collagens also possess non-helical, or non-collagen (NC), domains. Most collagens form supramolecular structures like fibrils or networks. The collagen superfamily can be divided into subfamilies based on the supramolecular structures formed by the different collagens. The collagens are named colla
Figure 1. Schematic figure of the loose connective tissue. Note that the dimensions of the different components are not to scale compared with each other.
XVIII are structurally similar, especially in the C-terminals. They have several triple-helical domains and are called multiplexins (multiple triple helix domains and interruptions) [20, 25]. Collagen type IV is present in basement membranes. Collagen type VIII is also associated with basement membranes and form hexagonal networks, as do collagen type X found in hypertrophic cartilage. Both the hexagonal networks formed by collagen types VIII and X, as well as the basement membrane structures formed by collagen type IV, are two-dimensional structures. Collagen types IV, VIII and X are therefore sometimes referred to as collagens forming sheets [18]. Collagen type VI form beaded filaments and collagen type VII anchoring fibrils. Collagen type VII interacts with collagen type IV and is involved in adherence of basement membranes to the epithelium, hence their name [18]. Collagen type XXVI was discovered through a yeast two-hybrid screening [26]. It lacks sequence and structural similarities with the existing collagen subfamilies and constitutes a separate subfamily.

There are a number of proteins that contain COL domain(s) but are not defined as collagens. The obvious question to ask is: What define collagens? Brown and Timpl defined collagens as large structural extracellular proteins with COL domain(s) [27]. This definition rightfully place proteins like acetylcholinesterase outside the collagen superfamily. However, according to the definition by Brown and Timpl the membrane collagens types XIII, XVII, XXIII and XXV should not belong to the collagen superfamily. There have not been any exact rules for distinguishing between collagens and collagen-like proteins. Perhaps some of the collagens should more rightfully belong to the group of collagen-like proteins or some of the collagen-like proteins should be defined as collagens.

**Fibronectin**

Fibronectins (FNs) are extracellular and plasma proteins that participate in a number of biological processes, such as cell migration, proliferation and differentiation. FN-null mice lack a vascular system and die before day E14.5 [28]. FNs are dimeric molecules composed of two large subunits, each around 250 kDa in size [7, 29]. The subunits are covalently linked in their C-terminal ends via disulfide bonds. The FN subunits are composed of three different types of repeating units, called I, II and III. Each subunit is composed of 12 type I, 2 type II and 15-17 type III units. Together these 29-31 units make up around 90% of the total FN sequence. The FN subunits are products of one gene, but three sites for alternative splicing leads to many splice variants [30]. EIIIA and EIIIB (also called EDA or EDI and EDB or EDII) are two type III repeats that are located between repeat III 7 and 8 and repeat III 11 and 12, respectively. These two repeats are sites for alternative splicing in the sense that they are either included or excluded. A variable region, V or IIICS (type III Connecting Segment), between type III repeats
14 and 15 is the third site for alternative slicing. The splicing of the V region is more complex than that of EIIIA and EIIIB. This region can be either partly or totally excluded, creating splice variants without V regions or with V regions of different sizes. The V region is, however, needed for secretion of FN from the cells and consequently, for secretion to occur, the V region has to be present in at least one of the subunits [31].

There are two major forms of FN, plasma FN and cellular FN [7, 29]. Plasma FN is soluble and has a concentration of 300 μg/mL in plasma. It is mainly produced by hepatocytes in the liver. Cellular FN is insoluble and is produced by many cell types including fibroblasts and endothelial cells. It assembles into fibrils and is part of the cell-produced matrix that surrounds cells. The EIIIA and EIIIB regions are almost always excluded from plasma FN whereas they are included in the cellular FN. The V region is present in most cellular FN subunits but only present in one of the two subunits in plasma FN dimers. Even though the importance of the V region for secretion of FN is known, the functions of EIIIA and EIIIB have not been completely elucidated. It has, however, been shown that splice variants expressed during wound healing contain EIIIA and EIIIB in contrast to splice variants expressed in normal adult tissues, where these regions are excluded [32]. The splice variants seen during wound healing are similar to those seen during early embryogenesis suggesting a role for EIIIA and EIIIB in FNs involved in tissue remodeling.

The binding sites for cells and other proteins on the FN molecules have been identified [29]. The essential binding site for FN can be found in the N-terminal type I repeats 1-5. Interactions between FN molecules are important for fibril formation. There are several binding sites for integrins along the FN molecule. The Arg-Gly-Asp (RGD) motif, a well-known cell-binding motif [33], is located in type III repeat 10. Type I repeats 6-9 and type II repeats 1-2 are binding sites for collagen, with a higher affinity for denatured than native collagens [34].

Integrins

Integrins are cell adhesion molecules that mediate contacts between cells and the ECM as well as cell–cell contacts [2, 35]. The name integrin is derived from their function to integrate the ECM with the cytoskeleton. Integrins are non-covalently associated heterodimeric transmembrane glycoproteins consisting of one α and one β subunit. There are 18 α and 8 β subunits known and according to genomic sequence studies by Whittaker and Hynes this is the complete set of mammalian integrin subunits [35]. The 18 α and 8 β subunits form αβ heterodimers and so far 24 integrin heterodimers have been described (Figure 2). Both the α and the β subunits consist of an N-terminal globular head region, a long stalk region that connects the globular
The integrin family of cell surface receptors. I domain containing α subunits are marked with a star.

head to the transmembrane part of the integrin and a short cytoplasmic tail [36]. The α subunits are somewhat larger (120-180 kDa) than the β subunits (90-110 kDa) with the exception of the β4 subunit found in hemidesmosomes, which is approximately 205 kDa in size due to a large cytoplasmic domain [2]. The N-terminal part of the α subunits consists of a seven-bladed β-propeller [37]. Nine of the α subunits have an inserted domain (I domain) between β sheets 2 and 3 of the β-propeller domain [37]. The integrin I domains show sequence similarities with von Willebrand factor A domains and are therefore sometimes referred to as A domains. In integrins lacking I domains, the β-propeller structure is directly involved in ligand binding. In I domain expressing integrins, the I domain is the major contributor to ligand binding. The β-propeller domain in these integrins are in some cases involved in ligand binding whereas they are not involved at all in others. The β subunits contain a domain that show weak sequence homology with the α subunit I domain and is therefore referred to as the I-like domain [37]. This domain interacts with the β-propeller domain of the α subunit and in integrins lacking I domains it seems to be directly involved in ligand binding. In integrins with I domains it seems to be indirectly involved in ligand binding. Different integrins have different ligand specificities. Many integrins bind more than one ligand and many ligands are recognized by more than one integrin. The collagen binding integrins α1β1, α2β1, α10β1 and α11β1 all bind collagen, but α1β1 and α2β1 in addition bind laminin [2, 38, 39]. The multi-specific integrin αVβ3 binds a number of different ligands containing the RGD motif like fibrinogen, vitronectin and fibronectin, but the
major fibronectin binding integrin seems to be the RGD dependent integrin \( \alpha 5\beta 1 \) [2].

Many integrins are present on the cell surface in an inactive form, and in order to bind ligand and to signal, the integrins have to be activated. There are two different schools regarding the mechanisms for integrin activation. One favors affinity modulation and the other avidity modulation [40, 41]. Electron microscopy pictures and crystal structure data of integrins indicate that the integrins can acquire two different three-dimensional structures [42]. One is a bent, inactive form where the extracellular stalk region is bent in a way that keeps the globular ligand-binding domain close to the cell membrane, the other an upright, active form where the globular domain of the integrin is more freely exposed [42-44] (reviewed in [37]). When there is a conformational change in the integrin structure that expose or open up binding sites for ligands, i.e. the affinity for the ligand is increased, one speaks of affinity modulation. However, it has been discussed whether a change in integrin conformation is a cause or a result of ligand binding [40]. Lateral movement of integrins in the cell membrane leading to clustering of integrins has been reported to increase ligand binding [45]. Clustering of integrins leading to increased ligand binding is referred to as avidity modulation. There is evidence of both affinity- and avidity-induced activation of integrins and existence of one does not exclude existence of the other. In fact, Hato and co-workers reported that clustering and conformational changes complement each other in signaling functions of the platelet integrin \( \alpha IIb\beta 3 \) [46]. Affinity modulation is sometimes also referred to as inside-out activation of integrins [2]. Integrins can mediate both inside-out and outside-in signaling [47]. One example of inside-out signaling is activation of integrin \( \alpha IIb\beta 3 \) that is present in an inactive form on circulating platelets [48]. Upon platelet activation, integrin \( \alpha IIb\beta 3 \) becomes activated from within the cell. After activation \( \alpha IIb\beta 3 \) binds fibrinogen, von Willebrand factor and fibronectin, which leads to adhesion of platelets to the vessel wall and platelet aggregation.

Integrin conformation on the cell surface is probably an equilibrium between the bent, inactive conformation and the up-right active conformation ([35] and figure 3). Ligand binding will stabilize the up-right integrin conformation and the equilibrium between inactive and active forms of the integrins is thereby shifted towards the active conformation [35]. Talin is a cytoplasmic protein that plays an important role in integrin activation [49, 50]. It binds both actin and integrin cytoplasmic domains. Rather than activating the integrin directly, talin stabilizes the up-right, active conformation of the integrins by binding to the cytoplasmic domain of the \( \beta \) subunit [51]. In this way the equilibrium is shifted towards the active integrin conformation and consequently, integrin affinity for ligand is increased. Talin has been shown to bind to the cytoplasmic domain of several \( \beta \) subunits and talin is therefore suggested to be a common step in integrin activation [52].
Integrins are present in the cell membrane in equilibrium between inactive and active conformations. Under normal conditions the equilibrium can be shifted more or less towards the inactive conformation depending on the integrin dimer. During integrin activation, the active conformation is stabilized, shifting the equilibrium towards the active conformation.

Integrins lack intrinsic enzymatic activity, but are able to recruit and activate tyrosine, serine and lipid kinases and in that way control a number of signaling pathways [53, 54]. When cells are cultured on a two dimensional (2D) ECM substrate, the cells link the ECM substrate to the cytoskeleton through integrins via intracellular connection proteins like paxillin and talin [55]. Integrins will cluster at these contact points and structures called focal adhesions (FA) are formed. A number of signaling molecules, including FAK, Grb2, protein kinase C (PKC) and mitogen-activated protein (MAP) kinases will be recruited to and/or activated at these adhesion sites. This is referred to as outside-in signaling and integrins are in this way able to influence a number of cellular processes like cell survival, proliferation and control of transcription to mention a few [35, 56]. There are different kinds of FAs depending on their size, what proteins they contain and where in the cell they are found [57]. One suggestion of how to define the different FAs is the definition of four different structures: focal complexes, focal adhesions, fibrillar adhesions and 3D adhesions [57]. Focal complexes are small FAs that are found in the periphery of migrating/spreading cells. They are associated with lamellipodia and filopodia and regulated by the small GTPases Rac and Cdc42 [58]. Focal complexes develop into larger focal adhesions as cell migration/spreading proceeds [58]. Focal adhesions associate with the ends of stress fibers in cells on a 2D substrate and can be found in the cell periphery as well as in more central parts of the cell. In contrast to focal complexes, focal adhesions are regulated by the small GTPase Rho [59-61]. Fibrillar adhesions are elongated focal adhesions found in the more central parts of the cell [62]. They appear later than focal adhesions and are formed as cell-produced fibronectin (FN) form fibrils [63]. Fibrillar adhesions are rich in integrin α5β1, which translocate from focal adhesions during formation of the fibrillar adhesions [62]. In contrast to focal complexes/adhesions,
which are rich in vinculin and paxillin and contains high levels of phosphotyrosine, fibrillar adhesions are rich in tensin and express only low levels of phosphotyrosine [64]. As FN fibrillogenesis proceeds, the cells become surrounded by a cell-produced three dimensional (3D) matrix and will adopt their matrix adhesions to the new environment [65]. 3D matrix adhesions are not as prominent as adhesion structures found in cells cultured on a 2D substrate. In contrast to what is seen on 2D substrates, integrin α5 and paxillin colocalize in 3D matrix adhesions and no auto-phosphorylation of FAK (focal adhesion kinase) at Y397 is seen [66]. 3D matrix adhesions formed as cells adhere to cell-derived 3D ECM matrices or to 3D collagen gels resemble the adhesion structures found in vivo [65]. The adhesion structures formed on 2D substrate can be seen as exaggerated forms of what is found in vivo, but it has to be remembered that there are differences in content between adhesion structures on 2D and 3D substrates and hence differences in signaling elicited from the respective structures [57, 63].

The adhesion structures described above are dynamic structures, an important quality during cell migration. In order to migrate, the cells need to be polarized with a leading edge where adhesion structures are formed and a rear end where adhesion structures are dissolved [67]. The leading edge is characterized by formation of broad lamellipodia and/or thin, long filopodia. The formation of these structures is dependent on re-organization of the actin cytoskeleton [68]. Integrins play an important role in migration. The protruding leading edge of the cell adheres to the underlying substrate at the same time as the rear end of the cell detaches from the substrate. In fibroblasts, the interactions between β1 integrins and the actin cytoskeleton in the rear end of the cell is often physically broken by the tension created as cell migration proceeds, leaving the integrins behind as the cell moves forward [69].

Many signaling pathways triggered by integrins are also triggered by growth factors. Furthermore, the presence of a crosstalk between integrins and growth factors in control of cellular functions has been suggested [70-72]. Miyamoto and coworkers showed an increase in extracellular-regulated kinase (ERK) activation after growth factor stimulation of fibroblasts if integrins were simultaneously engaged in ligand binding [73]. Similarly, Renshaw and coworkers showed that the mitogenic response triggered by growth factors depend on interactions between integrins and ECM components [74]. Direct physical interactions between integrins and ligand-activated growth factors have been described. Platelet-derived growth factor (PDGF) -β receptors, insulin receptors and vascular endothelial growth factor (VEGF) receptor-2 co-immunoprecipitate with integrin αVβ3, but not with β1 integrins [75-77]. The physical interactions between PDGF-β and VEGF-2 receptors with the αVβ3 integrins have been shown to depend on the extracellular domain of the β3 subunits [77]. In addition, the PDGF-β receptor is autophosphorylated in a ligand-independent manner when fibroblasts are adhering to ECM substrates, cultured in 3D collagen gels or stimu-
lated with anti-β1 integrin IgG [78]. Taken together these reports indicate the existence of a cross-talk between growth factors and integrins.

Integrin families
The 24 integrin heterodimers so far identified can be divided into three sub-families ([35] and Figure 2). The largest one, the β1 family, includes the collagen-binding α1β1, α2β1, α10β1 and α11β1 as well as the major fibronectin receptor α5β1. The β2 family is exclusively expressed on leukocytes. The third integrin sub-family, the αV family, includes αVβ1, αVβ3, αVβ5, αVβ6, αVβ8 and αIIbβ3 among which αVβ3 and αIIbβ3 have been most extensively studied [79]. αIIbβ3 is found on megakaryocytes and platelets, where it is involved in blood clotting. Platelets also express αVβ3, but many other types of cells, like fibroblasts and endothelial cells, also express αVβ3. αV integrins are important during development [80]. Expression of αVβ3 in normal tissue is low in most cases whereas expression levels are elevated in tumor tissues and sites of inflammation [81, 82]. αV null embryos develop normally until E9.5, but only 20% are born alive [83]. This is due to placental defects. The animals that are born alive die soon after birth, probably due to vascular defects in the brain. They suffer from severe hemorrhages in brains and intestines. Despite the fact that inhibitors of αV integrins impair angiogenesis [84], the vasculature develops normally in αV null embryos [83]. In contrast to the severe, lethal αV-null mutation, β3 null mice as well as β5 and β3/β5 null mice are viable and fertile [85-87]. The survival of the β3 null mice is, however, reduced. Both the litter size and the survival after birth is reduced compared to wild type. The smaller litter size is due both to fetal hemorrhage and defect placentas. β3 null mice show all symptoms of Glanzmann’s thrombasthenia, a human bleeding disorder caused by defects in the αIIb or β3 genes, and the reduced survival is caused by excessive bleeding in the GI tract [85].

Platelet-derived growth factor
As the name indicates, platelet-derived growth factor (PDGF) was first identified as a growth-promoting protein produced by platelets [88-90]. It is now known that a number of cells produce PDGF, including fibroblasts and macrophages [91, 92]. The members of the PDGF family are major mitogens for connective tissue cells such as fibroblasts [93]. The PDGFs consist of disulfide-bonded homo- and heterodimeric polypeptide chains. A-, B-, C- and D-polypeptide chains are products of four separate genes and form five different isoforms of PDGF: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD [94, 95]. PDGF-AA, -BB and -AB, as well as PDGF-CC and -DD, are synthesized in the endoplasmatic reticulum. The classical PDGFs
Figure 4. PDGF receptors and their ligands.

(PDGF-AA, -BB and -AB) are secreted in an active form whereas the novel PDGFs (PDGF-CC and -DD) are secreted in an inactive form and are activated extracellularly [95]. The PDGF dimers exert their actions by binding one of three PDGF tyrosine kinase receptors [93]. The PDGF receptors are homo- or heterodimers of two different receptor subunits, the α- and the β-subunit. PDGF-αα receptors are formed after stimulation with PDGF-AA, -BB, -AB and -CC, PDGF-αβ receptors by PDGF-AB and -BB and PDGF-ββ receptors by PDGF-BB and -DD (Figure 4). PDGF receptors are transmembrane proteins. The extracellular domain consists of five immunoglobulin-like domains of which the three outermost are responsible for ligand binding [96]. The intracellular tyrosine kinase domain with an inserted non-kinase sequence in the middle becomes autophosphorylated upon ligand binding [93, 97]. Fibroblasts express both α- and β-receptors, but usually higher levels of β-receptors [93]. The level of PDGF β-receptor expression on connective tissue cells is upregulated during inflammation and other pathological conditions. Vascular smooth muscle cells (SMC) in atherosclerotic plaques, rejected kidneys and chronic synovitis expressed higher levels of PDGF β-receptors than vascular SMC from the corresponding normal tissues [98]. It has also been shown that expression of PDGF β-receptors on fibroblasts from patients with severe asthma was higher than on fibroblasts from patients with mild/moderate asthma or no asthma [99].

Signaling pathways from both the α- and the β-receptors lead to membrane ruffling and loss of stress fibers in cultured cells after PDGF stimulation [100]. Formation of circular actin structures on the dorsal surface of the
cell is induced only by the activated \( \beta \)-receptor. Activation of the \( \beta \)-receptor stimulates chemotaxis whereas activation of the \( \alpha \)-receptor, depending on cell type, inhibits or stimulates chemotaxis. Furthermore, PDGF stimulates production of several extracellular matrix proteins, including fibronectin and collagen [101, 102].

**Inflammation**

Inflammation is the body’s response to tissue damage or infection caused by invasion of infectious agents, like bacteria or viruses. The clinical symptoms of inflammation are pain, redness, heat and swelling as described by the roman writer Aulus Celsus around year 30 AD. The purpose of inflammation is to limit the injured area, clear the body from infectious agents and restore damaged tissue. Swelling, or edema, is an early response to inflammation and is caused by vasodilating agents released by mast cells, leukocytes, macrophages and other inflammatory cells. Arachidonic acid (AA) is generated when inflammatory cells are activated and this leads to the production of prostaglandins (PGs) such as PGI\(_2\) (prostacyclin) and PGE\(_2\), two potent vasodilators [103]. Mast cells are found in the connective tissues and especially in the interface between tissues and the environment, like dermis of the skin and in mucosal surfaces of the lungs. Bacterial components activate mast cells, either by direct interactions or indirect by host products produced as a response to infection. This cause release of vasoactive agents from the activated mast cells [104]. Macrophages recruited to the site of inflammation are another source of vasodilating agents. They play an important role in the inflammatory response by releasing cytokines, like tumor necrosis factor (TNF)-\( \alpha \) and interleukin (IL)-1. TNF-\( \alpha \) and IL-1 are two important pro-inflammatory mediators. The presence of TNF-\( \alpha \) leads to a number of inflammatory responses in cells, e.g. in endothelial cells [105]. One effect of TNF-\( \alpha \) is activation of nuclear factor (NF)-\( \kappa B \), a transcription factor that plays an important role in inflammatory conditions by regulating transcription of cytokines and other inflammatory mediators [106]. The primary members of the IL-1 superfamily include pro-inflammatory IL-1\( \alpha \) and IL-1\( \beta \) and the anti-inflammatory IL-1 receptor antagonist (IL-1ra). IL-1\( \alpha \) is active both as an intracellular precursor and as a membrane-associated molecule but only slightly active as a secreted protein. IL-1\( \beta \), on the other hand, is only active in its secreted form. They both exert their actions by binding and activating one of the IL-1 receptors, IL-1RI or IL-1RII [107]. IL-1 stimulates production of prostaglandins and activates NF-\( \kappa B \) [106, 107].
Sepsis and septic shock

Bacteria or other pathogenic microorganisms that enter the bloodstream or internal tissues will trigger the immune response and can cause sepsis. Sepsis was defined as “the systemic inflammatory response syndrome that occurs during infection” at a consensus conference in 1991 [108]. To be characterized as sepsis, more than one of the following clinical symptoms should be present: 1) body temperature $>$38°C or $<$36°C, 2) heart rate $>$90 beats/min, 3) tachypnea (>20 breaths/min or hyperventilation) and/or 4) alteration in white blood cell count. Severe sepsis can lead to septic shock, which is characterized by fluid efflux from the circulation to the tissues leading to hypotension, besides the symptoms of sepsis [109]. The loss of fluid from the circulation and the resulting hypotension may result in multiple organ failure and death. Both gram-negative and gram-positive bacteria can cause sepsis and septic shock. Lipopolysaccharide (LPS) is the main pathogenic component in gram-negative bacteria whereas the gram-positive bacteria contain several pathogenic components that together can cause sepsis [109].

Gram-negative bacteria in sepsis

LPS is a component of the outer cell wall of gram-negative bacteria that is a potent activator of macrophages. LPS exerts its action by binding to toll-like receptors (TLR), mainly TLR-4 [110]. This family of receptors has gotten their name because they are structurally similar to the Drosophila melanogaster protein Toll. The cytosolic domain of the TLRs contains a TIR domain (reviewed in [111]). The TIR domain is a conserved region showing similarities to the cytosolic domain of the IL-1 receptor as well as the cytoplasmic part of the D. melanogaster Toll protein (Toll-IL-1 Receptor) [112]. For LPS to activate TLR-4, it has to bind to CD14 that is expressed on macrophages [113]. LBP (LPS-binding protein) facilitates association of LPS and CD14 by recruiting LPS to the cell surface [114]. The LPS-CD14 complex binds to TLR-4, which needs the association of an accessory protein, MD-2, to become efficiently stimulated [115].

Endothelial cells express TLR-4 [116], but they normally do not express membrane bound CD14 (mCD14). CD14 is, however, cleaved off cells expressing mCD14 and can therefore be found circulating in the plasma (sCD14). LPS can, together with sCD14, directly stimulate endothelial cells [117]. It is, however, debated whether the impaired vascular function seen during sepsis is caused by LPS acting directly on endothelial cells or indirectly by stimulating the release of cytokines from macrophages that in turn influence endothelial cell behavior [118-121].
**Gram-positive bacteria in sepsis**

Gram-positive bacteria do not contain LPS, but has been shown to induce a similar inflammatory response as gram-negative bacteria. Lipoteichoic acid (LTA), a cell wall component of gram-positive bacteria, has been shown to interact with LBP and CD14 in a way similar to LPS [122]. Peptidoglycan, another cell wall component of gram-positive bacteria, has also been implicated in the inflammatory response besides LTA, since on its own LTA does not induce a systemic response as strong as that seen for LPS [123]. De Kimpe and co-workers showed that LTA and peptidoglycan could work synergistically to induce septic shock in rats [124]. In contrast to LPS, gram-positive bacterial components seem to activate the immune system mainly by activating TLR-2 instead of TLR-4 [125]. Both LTA and peptidoglycan have been shown to activate endothelial cells [126]. These cells express TLR-2, indicating that gram-positive LTA and peptidoglycan can induce inflammation by acting on endothelial cells in a similar way as gram-negative LPS acting on endothelial cell-expressed TLR-4 [127].

**Streptococcus equi**

*Streptococcus equi* is a gram-positive Lancefield group C streptococcus and comprises three subspecies, subsp. *zooepidemicus*, subsp. *equi* and subsp. *ruminatorum*. Subsp. *zooepidemicus* is a horse commensal, but can cause disease under certain circumstances [128]. Subsp. *zooepidemicus* can also cause disease in humans [129, 130]. Subsp. *equi* is an obligate horse pathogen that causes a respiratory disease called strangles. It also infects donkeys and mules [131]. Subsp. *ruminatorum* was discovered recently and has so far only been isolated from sheep [132].

Extracellular proteins expressed by bacteria have been proposed to be important in establishing infection in hosts. MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) bind to host extracellular components and can in this way allow the bacteria to enter the host [133]. *S. equi*, like other bacteria, express a number of extracellular proteins that are either cell wall anchored or secreted. FNEB [134] is a cell wall anchored fibronectin-binding protein, whereas the fibronectin-binding SFS [135] and FNE [136] are secreted. There are, however, differences between the subspecies and whereas subsp. *equi* express the secreted FNE, the corresponding protein in subsp. *zooepidemicus*, called FNZ, is cell wall anchored [136, 137]. In fact, FNE is a truncated version of FNZ. The subsp. *equi* FNEB homologue in subsp. *zooepidemicus* is called FNZ2 [138]. CNE is a collagen-binding, cell wall anchored protein that is expressed by both subspecies [139]. Also SclC, a collagen-like cell wall anchored protein, can be found in both subspecies [140]. CNA is a collagen binding, cell wall bound protein of
Staphylococcus aureus that resembles CNE in S. equi. CNA has been shown to have structural similarities with integrin α1β1 in its collagen binding domain and bind collagen in a similar manner, as does integrin α1β1 [141]. ScIB, a Streptococcus pyogenes protein that is similar to the S. equi protein ScIC, has been shown to bind to integrin α2β1 via its collagen-like domain and in this way induce signaling similar to that induced by collagen [142].

![Diagram](image)

Figure 5. Schematic representation of the Starling forces. Modified from Reed et al. In: Connective Tissue Biology 1998.

Interstitial fluid pressure

Interstitial fluid pressure (P_{IFS}) is of importance for control of tissue fluid balance [143]. Fluid is constantly filtrated over the capillary wall and the process can be described by the Starling equation:

\[ J_V = K_F[(P_C - P_{IFS}) - \sigma(COP_C - COP_{IFS})] \]

where \( J_V \) is the net capillary fluid filtration, \( K_F \) is the capillary filtration coefficient, \( P_C \) is the hydrostatic pressure in the capillary (microvascular blood pressure), \( P_{IFS} \) is the interstitial fluid pressure (hydrostatic pressure in the interstitium), COP\(_C\) is the colloid osmotic pressure in the capillary (colloid osmotic pressure in plasma), COP\(_{IFS}\) is the colloid osmotic pressure in the
interstitium and $\sigma$ is the capillary reflection coefficient for proteins (Figure 5). If plasma proteins could pass freely across the capillary wall, $\sigma$ would be 0 and a capillary impermeable to protein would have a reflection coefficient equal to 1 [143]. The colloid osmotic pressures in plasma and interstitial fluid are, in experimental animals, approximately 20 and 12 mmHg, respectively [14]. The interstitial fluid pressure is approximately -1 mmHg in skin. The capillary hydrostatic pressure is around 10 mmHg. Under normal conditions there is a pressure imbalance across the vessel wall of 0.5–1 mmHg in skin and this imbalance will lead to a filtration of fluid over the vessel wall. The interstitium was previously thought to control the fluid balance in a rather passive manner. The interstitial fluid and colloid osmotic pressures counteract changes in the filtration and thereby regulate the interstitial fluid volume. Lately it has, however, been shown that the interstitium, or rather the connective tissue cells of the interstitium, under certain circumstances actively contribute to the control of fluid homeostasis [14].

As described in the beginning of this thesis, the connective tissue is composed of extracellular matrix (ECM) and connective tissue cells (Figure 1). The ECM consists of three major structural components: a fibrous network built up by rigid (mainly collagen) and elastic (e.g. elastin) fibers, linking proteins like laminin and fibronectin and space filling molecules like proteoglycans and hyaluronan [4]. The connective tissue has an intrinsic ability to take up fluid and swell. If a piece of loose connective tissue is put in a tube and allowed free access to physiological buffer it will swell and double its volume in around 48 h [144, 145]. The swelling is caused mainly by the proteoglycan and hyaluronan content of the tissue since pre-treatment of the tissue with chondroitinase ABC or hyaluronidase will inhibit swelling. In vivo, the connective tissue cells exert a tension on the fibrous network by binding the collagen fibers, thereby preventing the tissue from swelling [14]. The interactions between the cells and the fibrous network in vivo are mainly mediated by $\beta_1$ integrins. Injection of anti-$\beta_1$ integrin IgG lowers $P_{IF}$ in rat dermis [146]. Among the $\beta_1$ integrins, $\alpha_2\beta_1$ is of particular importance since injection of anti-$\alpha_2\beta_1$ integrin IgG in rat dermis lowers $P_{IF}$ whereas injection of anti-$\alpha_1\beta_1$ has no effect on $P_{IF}$ [147]. By releasing or increasing their tension on the collagen fibers, the integrins can actively control the volume of the ECM through controlling the $P_{IF}$ (Figure 6). The rapid formation of edema following acute inflammation is thought to involve regulation of $P_{IF}$ by connective tissue cells [14]. PDGF-BB and insulin have been shown to restore a lowered $P_{IF}$ in a phosphatidylinositol 3-kinase (PI3K) dependent manner [148, 149]. Work included in this thesis suggests that PDGF-BB restore $P_{IF}$ via a mechanism including integrin $\alpha V\beta 3$. Whether or not insulin uses $\alpha V\beta 3$ in restoration of a lowered $P_{IF}$ remains to be elucidated.
Collagen gel contraction

Fibroblasts seeded in, or on top of, a three-dimensional collagen gel will compact the gel by a process referred to as collagen gel contraction [150]. This process depends on collagen-binding β1 integrins and blocking of β1 integrins by antibodies will inhibit contraction [151]. In a relaxed free-floating collagen gel, the cells become quiescent and adopt a dendritic cell shape whereas cells in a stressed, attached gel are proliferative, have an elongated, bipolar morphology and develop stress fibers [152]. The different proliferative response of cells in stressed compared to relaxed gels can possibly be explained by their different response to growth factor stimulation. PDGF receptor autophosphorylation is reduced in relaxed collagen gels compared to stressed gels [153]. PDGF-BB and lysophosphatidic acid (LPA) stimulate contraction of relaxed collagen gels with similar kinetics, whereas LPA stimulates contraction of a stressed collagen gel more rapidly than PDGF-BB [154]. During collagen gel contraction the cells reorganize the collagen fibers [155]. It occurs when the cells try to migrate through the gel. In a stressed gel, the traction forces [156] exerted by the cells will organize the collagen fibrils in the same plane as the underlying restraint. Mechanical loading will therefore develop within the gel. Cells in a relaxed gel remodel the collagen fibrils at random, since there is little resistance to cellular force. This creates a mechanically unloaded gel [157].

Collagen gel contraction can serve as an in vitro model of in vivo wound contraction [152] and connective tissue cell control of tissue fluid homeostasis [14]. Morphologically and with respect to proliferative properties, cells in
a relaxed, free-floating collagen gel resemble cells found in normal dermis whereas cells in a stressed, attached gel resemble cells in granulation tissue [152]. Conditions that control collagen gel contraction of a relaxed, free-floating gel in vitro correlate with conditions controlling PIF in vivo. Interleukin-1 (IL-1) and prostaglandin E₁ (PGE₁) are examples of factors that inhibit collagen gel contraction whereas PDGF-BB and fetal calf serum will stimulate contraction [151, 158, 159]. In agreement with this, IL-1 and PGE₁ have been shown to lower PIF and PDGF-BB is able to restore a lowered PIF to normal levels [147, 159, 160].

There are several reports on a role of fibronectin (FN) in collagen gel contraction. In a study by Gillery and coworkers serum FN was shown to be important for collagen lattice contraction mediated by human skin fibroblasts [161]. Asaga and coworkers showed that cellular FN, but not plasma FN, was needed for fibroblast-mediated collagen gel contraction [162]. In contrast, several groups have reported that the process of cell-mediated collagen gel contraction is independent of FN [151, 163, 164].
Present investigations

Aim
The overall aim of this thesis work was to investigate mechanisms involved in tissue fluid homeostasis during inflammation.

The specific aims were:

- Investigating the role of integrin αVβ3 in control of interstitial fluid pressure in vivo and cell-mediated collagen gel contraction in vitro.

- Investigating the potential role of extracellular matrix-binding proteins from gram-positive cocci in modulating PIF by studying their effects on collagen gel contraction.

Methods
Two methods used in the present thesis work, collagen gel contraction and interstitial fluid pressure measurements, are central to these studies and are therefore described here in more detail.

Collagen gel contraction measurements
Assays to determine cell-mediated collagen gel contraction were performed in 96-well microtiter plates. Cells were mixed with a collagen solution to a final concentration of 1.2 mg/mL bovine dermal collagen type I and 100,000 cells/mL. 100 μL of the cell/collagen suspension was added to each well in the microtiter plate. The plates were left in 37°C for 1.5 h to allow the cell/collagen mixture to form gels. The gels were then detached from the walls of the well by careful but forceful addition of 100 μL of serum-free cell culture media. Factors investigated were either added to the flotation media or added to the cell/collagen suspension prior to addition to the microtiter plate. In the latter case, effectors were present when cell–collagen interactions were formed. The contraction was measured in free-floating gels by measuring the diameter of the cellular ring that will form in the gel soon
after flotation. The area of the gel was calculated and compared with the original gel area. Contraction is presented as percent of original gel area.

**Interstitial fluid pressure measurements**

Interstitial fluid pressure (Pif) can be measured *in vivo* using a micropuncture technique [165, 166]. Micropipettes with tip diameters of 3–5 μm connected to a servo-controlled unit with a Wheatstone bridge (an electrical circuit used to measure resistance) are inserted in dermal tissue. The counter-pressure created by a servo-controlled pump is recorded with a pressure transducer connected to a recorder. The pipette is filled with 0.5 M NaCl and hence has a greater conductivity than the interstitial fluid. The servo-controlled unit measures the conductivity of the solution in the pipette and the system is based on that a change in composition of electrolytes in the pipette tip will change the electrical resistance in the system. Since the pipette is part of a Wheatstone bridge, the imbalance in resistance will give rise to an error voltage. The pressure recorded is the counter-pressure applied by the pump to restore the pipette resistance.

The experimental setup consists of a pump, a servo-control unit, a micropipette connected to a micromanipulator and a recorder (Figure 7). The starting pressure is set to zero by placing the micropipette in 0.9 % NaCl at the level of the puncture site before start. Pressure is measured by puncturing the skin with the micropipette. A measurement is accepted when the following three criteria are fulfilled: (1) Feedback gain can be changed without changing the measured pressure, (2) applying suction to the pipette by the pump increases the resistance in the pipette. This ensures contact between the pi-
pette and the interstitial fluid, i.e. the pipette is open and (3) zero pressure
does not change during the measurement.

Results

Paper I

*Platelet-derived growth factor BB-mediated normalization of dermal inter-
stitial fluid pressure after mast cell degranulation depends on β3- but not
β1-integrins*

The aim of this study was to investigate the role of integrin αVβ3 in control
of interstitial fluid pressure (P_{IF}).

The connective tissue cells can actively control P_{IF}. Blocking of the β1 in-
tegrins by injection of polyclonal anti-β1 integrin IgG or monoclonal anti-
α2β1 integrin IgG lowers P_{IF} in dermis [147]. When the anti-β1 integrin
IgGs are injected together with PDGF-BB there is, however, no effect on P_{IF}
[147]. PDGF-BB also restores dermal P_{IF} to normal levels after it has been
lowered as a result of anaphylaxis induced by dextran [147] or C48/80, a
mast cell-degranulator [148]. The mechanism for this was, however, not
known. Data from our laboratory, and from others, [167, 168] have demon-
strated that integrin αVβ3 is involved in PDGF-BB-mediated stimulation of
collagen gel contraction *in vitro* and we therefore hypothesized that integrin
αVβ3 might be involved in PDGF BB-mediated control of P_{IF}.

In Paper I we showed that PDGF-BB restored a lowered P_{IF} via a mecha-
nism involving integrin αVβ3. In agreement with earlier results, anti-β1
integrin IgM caused a lowering of P_{IF} in normal dermis and PDGF-BB was
able to restore a P_{IF} that had been lowered after mast cell degranulation by
C48/80. Anti-β1 integrin IgM had no effect on the ability of PDGF-BB to
restore a lowered P_{IF}, since PDGF-BB was able to restore P_{IF} in the presence
of anti-β1 integrin IgM. Injection of anti-β3 integrin IgG had no effect on P_{IF}
when it was injected in normal dermis. However, PDGF-BB was not able to
restore a lowered P_{IF} in the presence of anti-β3 integrin IgG or an αVβ3-
specific inhibitor. Furthermore, PDGF-BB could not restore a lowered P_{IF} in
integrin β3 null mice. Collagen gel contraction has been shown to function as an *in vitro* model for control of P_{IF} *in vivo*. In the present studies we
showed that collagen gel contraction mediated by αV-negative cells, in con-
trast to wild type cells, were not stimulated by PDGF-BB in the presence of
anti-β1 integrin IgG.

In summary we show that P_{IF} under normal conditions is dependent on β1
integrins. During anaphylaxis, the β1 integrins are non-functional and in-
tegrin αVβ3 is of importance for PDGF-BB-stimulated normalization of P_{IF}.
Paper II

*A fibronectin-binding protein from Streptococcus equi binds collagen and modulates cell-mediated collagen gel contraction*

The aim of this study was to investigate the effects of a bacterial protein from *Streptococcus equi* on cell-mediated collagen gel contraction.

*S. equi* subspecies *equi* is a pathogen that causes respiratory diseases in horse. Subsp. *zooepidemicus* also causes infection in horse, but is less pathogenic than subsp. *equi*. Both subspecies produce a number of fibronectin (FN)-binding proteins. *S. equi* subsp. *zooepidemicus* expresses a cell wall anchored FN-binding protein, FNZ. The corresponding protein in *S. equi* subsp. *equi* is called FNE. It is homologous to the N-terminal part of FNZ but, in contrast to FNZ, it is secreted. The protein used in Paper II was derived from the N-terminal part of the subsp. *zooepidemicus* protein FNZ and is identical to the subsp. *equi* protein FNE with an additional 40 amino acids. This protein was called FNZN. Antibodies to FNZN have protective effects against subsp. *equi* infection [169]. This means that anti-FNZN antibodies have protective effects against infections involving the protein FNE (from the more pathogenic subsp. *equi*) even though the protein used for vaccination is derived from the subsp. *zooepidemicus* protein FNZ.

In Paper II we showed that FNZN stimulated C2C12-mediated collagen gel contraction via an integrin αVβ3-dependent mechanism. C2C12 is a murine myoblastic cell line lacking collagen binding β1 integrins [39]. We showed that FNZN, besides binding to FN, bound native collagen type I. In addition, FNZN bound to C2C12 cells, both in solution and when the FNZN protein was immobilized on cell culture plastic dishes. C2C12 cells do not express any collagen binding integrins. The observed binding of FNZN both to collagen and C2C12 cells opened up the possibility that FNZN stimulated contraction by forming a link between the collagen fibrils and the cells. This seemed, however, not to be the case since FNZN could not initiate binding of C2C12 cells to immobilized collagen. FNZN had no effect on melting temperature of collagen suggesting that it did not influence the triple helical conformation of the collagen molecules. The possibility that FNZN relaxes the triple helix, thereby exposing cryptic RGD motifs within the collagen molecules that could serve as binding sites for integrin αVβ3 on the cell surface thus seems less likely. In fact, the binding of C2C12 cells to FNZN seemed independent of integrin αVβ3 since an αV-inhibitor could not block binding of cells to immobilized FNZN. A possible mechanism for FNZN-stimulated contraction by C2C12 cells includes the fact that FNZN, besides being able to bind collagen and cells, also binds FN. FNZN could be included in a complex with cellular FN and collagen on the cell surface thereby creating a support for αVβ3-mediated contraction.
Even though further studies are needed to elucidate the mechanism for FNZN-stimulated collagen gel contraction by C2C12 cells, it is of potential clinical importance that a secreted bacterial protein has the ability to modulate collagen gel contraction that is an in vitro model of in vivo control of PIF. This suggests that FNZN could be involved in control of fluid homeostasis during infection.

Paper III

*Effects of streptococcal extracellular matrix-binding proteins on αVβ3-directed collagen gel contraction*

The aim of this study was to investigate effects of extracellular bacterial proteins in collagen gel contraction and to elucidate mechanisms involved in αVβ3-mediated collagen gel contraction.

In the present studies we took advantage of two cell lines that do not express any collagen binding integrins, the C2C12 cell line used in Paper II and FN null mouse embryonic fibroblasts (MEFs). Transfection of wildtype C2C12 cells with α2 cDNA generates C2C12 cells expressing α2β1 (C2C12-α2) as the only collagen binding integrin [39]. These cells allow for a comparison between cells expressing no collagen binding integrins or α2β1 as the only collagen binding integrin.

In Paper III we showed that C2C12 cells mediated collagen gel contraction in a process dependent on integrin αVβ3, but only after activation of the cells, e.g. with PDGF-BB. Stimulation of C2C12 cells with PDGF-BB, clustering of β1 integrins by addition of anti-β1 integrin IgM or addition of human FN (HFN) or FNZN to the gels all induced αVβ3-mediated contraction. PDGF-BB in combination with anti-β1 integrin IgM, HFN or FNZN induced a synergistic stimulatory effect on C2C12 mediated contraction. Collagen gel contraction mediated by FN null MEFs was synergistically stimulated by anti-β1 integrin IgM or HFN in combination with PDGF-BB. No stimulatory effect was, however, seen when FN null MEFs were stimulated with FNZN, neither alone nor in combination with PDGF-BB. These results suggest that integrin αVβ3 regulate collagen gel contraction by FN-dependent and -independent mechanisms.

Among a panel of extracellular proteins from *S. equi* only one, besides FNZN, modulated collagen gel contraction by C2C12 cells. The collagen binding protein CNE inhibited αVβ3-mediated collagen gel contraction, but had no effect on α2β1-mediated contraction.

The PDGF-BB-stimulatory effect on C2C12-mediated contraction was dependent on the MAP kinase ERK, whereas the stimulatory effect of anti-β1 integrin IgM was not. The synergistic stimulatory effect of C2C12-mediated contraction by PDGF-BB in combination with anti-β1 integrin IgM
was ERK-independent. This indicates that αVβ3 can mediate contraction via different, independent signaling pathways.

C2C12-α2 cells mediated contraction via integrin α2β1. Integrin αVβ3 was only able to mediate contraction in cells that did not express integrin α2β1 or in cells where integrin α2β1 function was blocked. This was shown by the fact that unless integrin α2β1 was blocked by anti-integrin β1 IgM, addition of anti-integrin β3 IgG had no effect on C2C12-α2-mediated contraction. Integrin α2β1 mediated contraction via an ERK-independent mechanism.

In summary, extracellular bacterial proteins modulated αVβ3-directed collagen gel contraction but not α2β1-directed contraction. Integrin αVβ3 mediated contraction via FN-dependent and -independent mechanisms.
Discussion and future perspectives

The data presented in this thesis shows a previously unrecognized role for integrin αVβ3 in control of tissue fluid homeostasis. Lowering of PIF during the early stages of inflammation results in the driving force for edema formation. In Paper I we show that PDGF-BB counteract such a lowering of PIF via an integrin αVβ3-dependent mechanism.

In Papers II and III of this thesis we show effects of extracellular bacterial proteins from gram-positive Streptococcus equi on integrin αVβ3-directed collagen gel contraction. That these proteins modulated in vitro collagen gel contraction makes it possible to speculate that these proteins may influence PIF in vivo. Extracellular bacterial proteins are involved in host response to infection [133]. In Paper I we showed that β1 integrins control PIF in normal dermis whereas αVβ3 integrins are involved in PDGF-BB-mediated control of PIF during anaphylaxis. The results from Paper II and III indicate that the extracellular bacterial proteins could influence PIF, and their effect on αVβ3-mediated collagen gel contraction suggest a role during inflammatory reactions. Investigation of potential effects of these extracellular bacterial proteins on PIF in vivo would be most interesting. If extracellular, bacterial proteins are involved in αVβ3-directed control of PIF, this could potentially be of clinical importance. Sepsis and septic shock are severe, and sometimes lethal, conditions caused by gram-negative, as well as gram-positive, bacteria. Elucidating mechanisms for how bacterial proteins modulate PIF could lead to more effective treatment of sepsis and septic shock.

Solid malignancies are characterized by an elevated PIF [170]. The high tumor PIF may impair the exchange of fluid within the tumor. This can lead to poor delivery of drugs to the tumor tissue. Data from our group shows that lowering of the tumor PIF in combination with chemotherapy increases the efficiency of the treatment [171]. In this study by Salnikov and co-workers, tumor PIF was lowered by administration of PGE1, a substance that inhibits collagen gel contraction in vitro and lowers PIF in vivo [159]. Similar results have been achieved by lowering of tumor PIF by administration of the PDGF receptor tyrosine kinase inhibitor STI571 (Gleevec) [172]. That PDGF is involved in the elevated PIF in solid tumors is in agreement with the effects of PDGF on restoration of a lowered PIF in normal dermis. Based on the results in this thesis it is tempting to speculate that also integrin αVβ3 is involved in the elevated tumor PIF. Further investigations should be performed to elucidate the potential role of integrin αVβ3 in the elevated PIF in...
tumors. One way of doing that is to take advantage of the integrin β3 null mice from Paper I. Integrin β3, as well as β3/β5, null mice support tumor growth. Measuring tumor $P_{IF}$ in tumors in integrin β3 or β3/β5 null mice would help elucidating the effects of αV integrins in the elevated $P_{IF}$ seen in tumors. It would also be interesting to investigate effects of the PDGF receptor inhibitor STI571 on tumor $P_{IF}$ in these animals.

This thesis furthermore includes investigation of the differences between integrin α2β1- and αVβ3-mediated collagen gel contractions. Whereas integrin αVβ3 was unable to mediate contraction unless the cells were stimulated by e.g. PDGF-BB, integrin α2β1-mediated contraction proceeded well in the absence of stimulators. Our data suggests that αVβ3-mediated contraction was of importance only in cells lacking integrin α2β1 or when integrin α2β1 function was blocked. This is in accordance with results from $P_{IF}$ measurements where integrin αVβ3 seemed to be important in tissue fluid homeostasis during inflammatory reactions when integrin β1 function is disturbed. Integrin αVβ3 was furthermore shown to mediate contraction via both FN-dependent and -independent mechanisms, whereas α2β1-mediated contraction was FN-independent. Since integrin αVβ3 does not bind triple helical collagen, FN could possibly serve as a link between the cells and collagen. The mechanism for FN-independent αVβ3-mediated collagen gel contraction is not known. The collagen triple helix contains cryptic RGD motifs that are hidden within the helix. Leikina and coworkers reported that the triple helix is partly denatured at body temperature [173]. This would result in exposure of the otherwise cryptic RGD motifs and allow for integrin αVβ3 to bind collagen, a possible mechanism that needs further investigation.

The integrin β3 null mice used in Paper I allow interesting investigations concerning the importance of this integrin in control of tissue fluid homeostasis. Insulin was shown to normalize a lowered $P_{IF}$ in a similar way as PDGF-BB [149]. However, it is not known whether or not insulin restores $P_{IF}$ via an integrin αVβ3-dependent mechanism. The β3 null mice allows for investigation of this mechanism. Furthermore, it would be interesting to investigate the effects of edema in these mice. One could imagine that mice lacking β3 integrins are more susceptible to edema formation as well as have a reduced ability to counteract edema.
Concluding remarks

The results presented in this thesis could potentially be of clinical importance in treatment of sepsis and septic shock. We show a novel mechanism for integrin αVβ3 in control of tissue fluid homeostasis during inflammation. In addition, we show that extracellular, bacterial proteins from gram-positive bacteria modulate cell-mediated collagen gel contraction. This indicates a possible role for these proteins in control of tissue fluid homeostasis. The latter is, however, an assumption that needs further investigation. Together, these results may help elucidating mechanisms involved in establishing sepsis and septic shock.
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“What you do is not so important, it is who you do it with that matters”. 

34
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Tack för ALLT!
References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)