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Cell-Surface Association between Progelatinases and β_2 Integrins: Role of the Complexes in Leukocyte Migration

MICHAEL STEFANIDAKIS

Department of Biological and Environmental Sciences, Division of Biochemistry and
Turku Graduate School "In vitro diagnostics", Faculty of Biosciences,
University of Helsinki,
Finland

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Supervised by:

Docent Erkki Koivunen
Department of Biological and Environmental Sciences
Division of Biochemistry
University of Helsinki
Finland

and

Professor Carl G. Gahmberg
Department of Biological and Environmental Sciences
Division of Biochemistry
University of Helsinki
Finland

Reviewed by:

Professor Jari Ylänne
Department of Biological and Environmental Sciences
University of Jyväskylä
Finland

and

Docent Jukka Westermarck
Center for Biotechnology
University of Turku & Abo Academi University
Finland

Opponent:
Professor Antti Vaheri
Department of Virology
Haartman Institute
University of Helsinki
Finland

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Integrins: connecting MMPs

To my Family

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REFERENCES

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals I-IV:

I

Stefanidakis, M., Björklund, M., Ihanus, E., Gahmberg, C. G., and Koivunen, E. Identification of a negatively charged peptide motif within the catalytic domain of progelatinases that mediates binding to leukocyte β_2 integrins. *Journal of Biological Chemistry*, **278**, 34674-84, 2003.

II

Stefanidakis, M., Ruohtula, T., Borregaard, N., Gahmberg, C. G., and Koivunen, E. Intracellular and cell surface localization of a complex between $\alpha_M\beta_2$ integrin and promatrix metalloproteinase-9 progelatinase in neutrophils. *Journal of Immunology*, **172**, 7060-68, 2004.

III

Björklund, M., Aitio, O., **Stefanidakis, M.**, Suojanen, J., Salo, T., Sorsa, T., Koivunen, E. Stabilization of the activated $\alpha_M\beta_2$ integrin by a small molecule inhibits leukocyte migration and recruitment. *Biochemistry*, In press, 2006.

IV

Stefanidakis, M., Gahmberg, C. G., Jaalouk, D., Pasqualini, R., Arap, W., and Koivunen, E. Disruption of a cell surface MMP-9/β_2 integrin interaction inhibits leukemia medullary extravasation and extramedullary tissue invasion. Submitted, 2006.

ABBREVIATIONS

ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with a thrombospondin motif
α_2-M	α_2-macroglobulin
AML	acute myelocytic leukemia
APMA	aminophenyl mercuric acetate
APC	antigen presenting cell
BFGF	basic fibroblast growth factor
BPI	bactericidal/permeability-increasing protein
CTT	gelatinase inhibitor peptide CTTHWGFTLC
DDGW	$\alpha_{M/L}$ I domain ligand peptide ADGACILWMDDGWCGAAG
ECM	extracellular matrix
EGF	epidermal growth factor
EM	electron microscopy
EMMPRIN	extracellular matrix metalloproteinase inducer
FAK	focal adhesion kinase
fMLP	N-formyl-methionyl-leucylphenylalanine
FnIII	fibronectin type III
GST	glutathione-S-transferase
ICAM	intercellular adhesion molecule
IgSF	immunoglobulin superfamily
LAD	leukocyte adhesion deficiency
LFA	leukocyte function-associated antigen
LPS	lipopolysaccharide
LRP	low-density lipoprotein receptor-related protein
MAbs	monoclonal antibodies
MadCAM	mucosal addressin cell adhesion molecule
MAPK	mitogen-activated protein kinase
MCP-3	monocyte chemotactic protein-3
MIDAS	metal ion-dependent adhesion site
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NGAL	neutrophil gelatinase-associated lipocalin
NMR	nuclear magnetic resonance
PECAM	platelet-endothelial cell adhesion molecule
RECK	reversion-inducing cysteine rich protein with kazal motifs
RME	receptor-mediated endocytosis
ROI	reactive oxygen intermediate
SDF-1	stromal-cell derived factor-1
TNF-α	tumor necrosis factor-alpha
tPA	tissue-type plasminogen activator
uPA	urokinase type plasminogen activator
uPAR	urokinase type plasminogen activator receptor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA	very late antigen
TIMP	tissue inhibitor of metalloproteinases

ABSTRACT

Leukocyte motility is known to be dependent on both β_2-integrins and matrix metalloproteinases MMP-2/-9 or gelatinases, capable of mediating leukocyte adhesion and the proteolysis needed for invasion, respectively. We have used phage display technology to identify peptide sequences interacting with the α_M integrin I domain, an about 200 amino acid residue sequence known to be responsible for ligand binding in β_2 integrins. One of the peptides contained a sequence very similar to the conserved DELW(S/T)LG sequence found in MMP-2 and –9. In several binding, migration and mutation analysis studies, we showed that the integrin recognition sequence mapped to the MMP catalytic domain, specifically bound to the α_M I domain, and it inhibited migration of leukocytes *in vitro*. Subcellular fractionation experiments revealed that the proMMP-9/$\alpha_M\beta_2$ complex was formed intracellularly and could be translocated to the cell surface upon cell activation. This interaction was efficiently blocked by a peptide sequence derived from the catalytic domain of MMP-9. Also, a novel small-molecule ligand to the α_M I domain, identified by screening a combinatorial library, inhibited DDGW-phage binding to the I domain and reduced leukocyte infiltration to an inflammatory site *in vivo*. The concept that MMPs associate with integrins, as well as its importance in some physiological and pathological conditions has been advanced previously but has not been examined on leukocytes.

Gelatinases not only play an important role in cell migration, tissue remodelling and angiogenesis during development, but are also involved in the progression and invasiveness of many cancers, including leukemias. We showed that MMP-9 association with β_2 integrins seems to play an important role in leukemia growth and dissemination *in vivo*, as inhibition of complex formation significantly improved the survival of mice that developed leukemia. These findings suggest that the integrin/MMP-9 complex may serve as a functional target for intervention in human acute leukemias.

REVIEW OF THE LITERATURE

LEUKOCYTE ADHESION AND MIGRATION

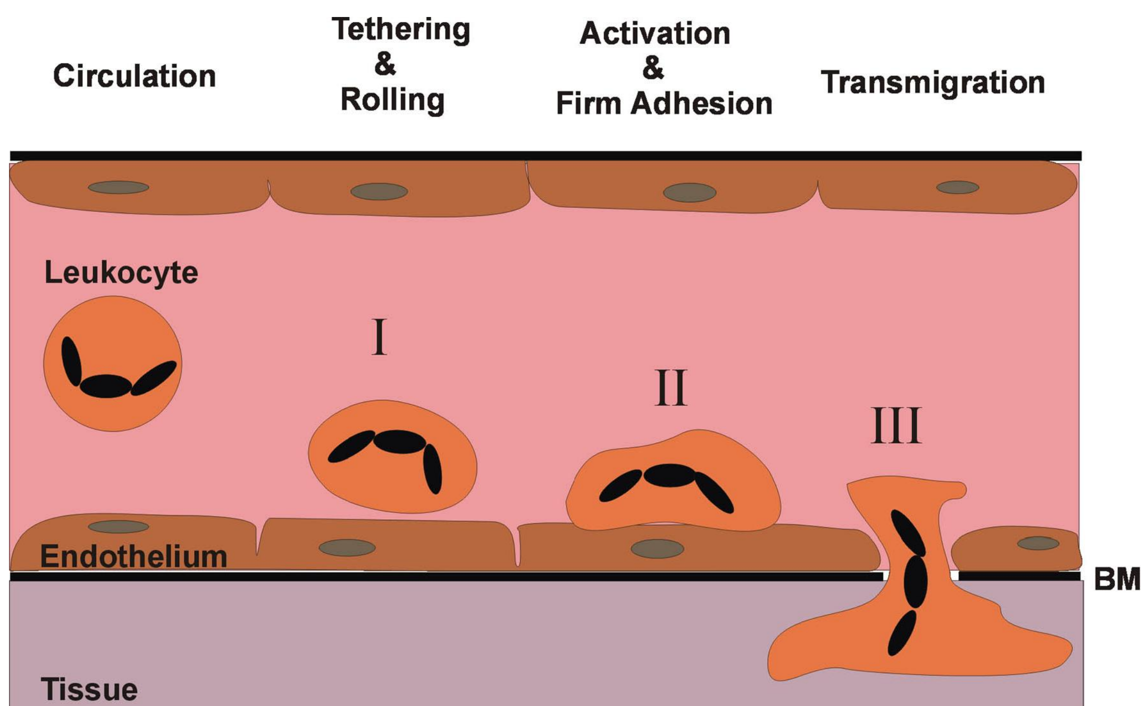
THE LEUKOCYTE MIGRATION CASCADE

Leukocytes or white blood cells (WBC) are bone marrow-derived cells and principal components of the immune system. They circulate in the bloodstream as passive, non-polarized cells and function by destroying “nonself” substances, including invading microbes, bacteria, and viruses. Over the past two decades, much progress has been made towards elucidating the molecular basis of leukocyte migration from the bloodstream to the tissues (reviewed in Butcher, 1991; Springer, 1995; Carlos and Harlan; 1994; von Andrian and Mackay, 2000; McIntyre et al., 2003). Recruitment of neutrophils from the blood to the inflamed tissues requires a sequence of adhesion and activation events which are mediated by several adhesion molecules, including mainly selectins (that bind to their carbohydrate-based ligands) and integrins (that interact with cell adhesion molecules or CAMs) (Vestweber and Blanks, 1999; von Andrian and Mackay, 2000) (Figure 1).

At least four steps of adhesion and activation events are required for a succesful extravasation of leukocytes from the vascular lumen to the tissues: (I) “Thethering and rolling” is the initial and essential event in leukocyte recruitment. It describes a process of weak adhesive interactions between the surfaces of the neutrophil and the endothelial cell, largely mediated by three members of the selectin family and their highly glycosylated ligands. Weak adhesive interactions between selectins and their ligands tether neutrophils to the vascular endothelium, and under shear flow, causes them to crawl along it. Such interactions can also initiate signals which promote the opening of cell-cell junctions, allowing leukocytes to pass between tissue (Johnson-Leger et al., 2000) or within (transcytosis; Middleton et al., 1997) endothelial cells in order to reach the underlying tissue.

Selectins are a family of cell surface adhesion glycoproteins, which share a conserved sequence and named according to their main expression sites. L-selectin (LECAM-1, CD62L) is expressed exclusively on leukocytes, whereas E-selectin (ELAM-1, CD62E) and P-selectin (GMP-140, CD62L) are expressed on endothelial cells. P selectin is also found in platelets.

(II) “Slow rolling and activation” is associated with increased integrin avidity, which can be elicited by soluble and/or membrane bound chemokines or other chemotactic compounds, such as bacterial peptides, the platelet activating factor (PAF) or leukotriene B4.



Functions	Leukocytes (receptors)	Endothelium (ligands)
I	L-selectin PSGL-1,CD24, sLeX ESL-1, CLA, L-selectin, SSEA-1	CD34, sLeX, PSGL-1, GlyCAM, MAdCAM-1, CD14 P-selectin E-selectin
II	$\alpha_{L/M/X/D}\beta_2$ $\alpha_4\beta_1$ $\alpha_4\beta_7$ Chemokine receptors	ICAMs, VCAM-1, iCb3, FG VCAM-1, ECM VCAM-1, MAdCAM-1, FN Chemokines
III	PECAM-1 $\alpha_L\beta_2$ $\alpha_M\beta_2$ $\alpha_4\beta_1$ PECAM-1 JAM-A JAM-C CD99	PECAM-1 ICAMs, JAM-A ICAMs, JAM-C (on platelets) VCAM-1, ECM, CD44, JAM-B PECAM-1, $\alpha_V\beta_3$ JAM-A ? JAM-C CD99

Figure 1. Adhesion molecules involved in different steps of the leukocyte adhesion /migration cascade. CD, cluster of differentiation; CAM, cell adhesion molecule; ICAM, intercellular CAM; CLA, cutaneous lymphocyte antigen; ECM, extracellular matrix; ESL, E-selectin ligand; MAdCAM, mucosal addressin CAM; PSGL, P-selectin glycoprotein ligand; SSEA, sialyl stage-specific embryonic antigen; VCAM, vascular endothelial CAM; sLeX, sialyl Lewis X; FG, fibrinogen; JAM, junctional adhesion molecule; PECAM-1, platelet-endothelial-cell adhesion molecule-1.

Chemokines are a large family of extracellular signaling molecules, capable of signaling through G-protein-coupled receptors and being key regulators of the immune system (reviewed in Mackay, 2001). They are also known to function as modulators of adhesion events mediated by integrins and selectins, and to regulate the order and timing of integrin adhesions. Treatment of cells with several chemokines promotes β_2-integrin-mediated adhesions to ICAMs by increasing β_2-integrin clustering and affinity in leukocytes (Goda et al., 2000). However, several chemokines induce adhesion through activation of $\alpha_4\beta_1$, another major leukocyte integrin, but this activation is often followed by inactivation and leukocyte detachment (Weber et

al., 1996; Grabovsky et al., 2000), suggesting that chemokines may shift leukocytes from $\alpha_4\beta_1$- to β_2-mediated adhesions. Moreover, in order for these adhesive events to occur, it has been suggested that chemokines should be first immobilized by various proteoglycans on the luminal endothelial surface and presented in a bound form to neutrophils (Rot, 1992; Tanaka et al., 1993).

(III) "Firm adhesion" and "Transmigration" are both mediated mostly by integrins and their ligands. Firm adhesion of neutrophils to activated endothelium is a step required for further transendothelial migration and disruption of the tight barriers formed by endothelial junctional proteins. It appears that $\alpha_M\beta_2$-integrin is one of the key molecules responsible for firm adhesion of neutrophils to the vascular endothelium *in vivo* (Bunting et al., 2002), as treatment of animals with anti-β_2 antibodies resulted in inhibition of cell adhesion after an inflammatory stimulus (Arfors et al., 1987).

To date, despite the intense studies performed by several investigators using techniques such as intravital, fluorescence, and electron microscopy, it still remains unclear which exact pathway neutrophils use to migrate out of blood vessels. These techniques allowed the elucidation of two migration pathways: (1) neutrophil migration at intercellular junctions (paracellular migration) (Marchesi and Florey, 1960; Burns et al., 2000; Shaw et al., 2001) and (2) neutrophil migration through an endothelial cell body (transcellular migration) (Feng et al., 1998). For a successful paracellular migration, neutrophils need to cross the endothelial cell-cell junctions formed by a large number of proteins, including the vascular endothelial (VE)-cadherin, members of the junction adhesion molecule (JAM) family, claudins, CD99, occludin and PECAM-1 (Figure 1). Antibodies against PECAM-1 dramatically decreased transendothelial migration, both *in vitro* (Muller et al., 1993; Christofidou-Solomidou et al., 1997) and *in vivo* (Vaporciyan et al., 1993; Mamdouh et al., 2003). In addition, chemoattractant gradients play essential roles in providing routes to leukocytes for polarized migration through the endothelium, and through the ECM into the tissue (Foxman et al., 1997).

NEUTROPHIL FUNCTION AND ADHESION DURING INFLAMMATION

Neutrophils, also known as polymorphonuclear leukocytes (PMNs) originate from stem cells in the bone marrow. They represent 60-70% of the total circulating leukocytes and are the first cells to be recruited to the sites of infection or injury within minutes to hours after maturation, forming a primary defense against infectious agents or "foreign" substances that invade our body's physical barriers. The initiation of an inflammatory response involves three major steps: (1) increased blood flow by dilation of capillaries; (2) escape of plasma proteins from the bloodstream; (3) and extravasation of neutrophils through the endothelium and accumulation at the site of injury. Elimination of invading microorganisms is accomplished by phagocytosis, generation of reactive oxygen metabolites, as well as through release of proteolytic enzymes and microbicidal substances, all stored in intracellular granules of mature PMNs (Bainton, 1999).

The main functions of neutrophils describe adhesion, extravasation, chemotaxis, phagocytosis, and production of oxidative agents. Like all leukocytes, these functions can be triggered by appropriate stimuli and the synergistic action of different adhesion molecules that are present on the surface of both neutrophils

and activated endothelial cells (reviewed in Zimmerman et al., 1992; Carlos and Harlan, 1994). Interactions of neutrophils with the activated endothelium have been extensively studied either under static conditions or under physiological conditions (flow shear forces). A new technique was developed to study neutrophil arrest in inflamed venules *in vivo*, called leukocyte tracking (reviewed in Ley, 2002). Neutrophil tethering and capture has been shown to be mediated by P-selectin-binding to its ligand PSGL-1; neutrophil activation by chemokines, such as IL-8; and firm adhesion by ICAM-1-binding to $\alpha_L\beta_2$ and $\alpha_M\beta_2$ integrins (Divietro et al., 2001). Chemokines, capable of triggering rapid arrest of T cells, B cells, and monocytes on endothelial cells under physiological conditions include SLC/CCL21, RANTES, and SLC/CCL21 or SDF-1/CXCL12, respectively. Unlike other leukocytes, arrest chemokines for neutrophils have been much difficult to define, even though the neutrophil adhesion cascade has been studied longer and by more groups. In certain *in vitro* systems, rapid neutrophil adhesion can be triggered by a single chemoattractant, such as IL-8, the platelet activating factor (PAF), complement C5a, formyl peptides, and leukotriene LTB4. However, the presence of a single chemoattractant has little effect on P-selectin-dependent neutrophil rolling and chemoattractant-dependent activation in most inflammatory models *in vivo*. In contrary to naive T cells, neutrophils need multiple inputs for full activation rather than a single arrest chemokine, mediated by additive or even synergistic signals through G-protein-coupled receptors, Fc receptors, and inflammatory adhesion molecules (Ley, 2002). Finally, chemokines are responsible for changes in neutrophil morphology, from a spherical to a polarized motile shape with a leading edge and a uropod that concentrates a great number of adhesion molecules, known to be required for PMN rolling and chemotaxis (del Pozo et al., 1995).

Activation of neutrophils can be achieved with nanomolar concentrations of phorbol esters *in vitro* (Patarroyo et al., 1985). Such type of stimulation can lead to rapid mobilization of different subsets neutrophil cytoplasmic granules, as well as, secretory vesicles for exocytosis (Kjeldsen et al., 1992), whereas similar concentrations of fMLP can only induce discharge of secretory vesicles (Sengelov et al., 1993). Binding of chemoattractants to their corresponding G-protein-coupled receptors leads to the activation of phospholipase C, which in turns, cleaves phosphoinositol (4,5) biphosphate (PIP2) into inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG). IP3 induces elevation in intracellular Ca^{2+} levels, whereas DAG activates protein kinase C (PKC). Increase in intracellular Ca^{2+} levels results in integrin activation via inside-out signaling (Altieri et al., 1992; van Kooyk et al., 1993) and when Ca^{2+} reaches concentrations as high as 40-50 nM, it induces complete release of secretory vesicles from neutrophils (Nüsse et al., 1998). Apparently, neutrophil degranulation can also be triggered by elevations in intracellular Ca^{2+} levels, especially after L-selectin and $\alpha_M\beta_2$ integrin engagement (Ng-Sikorski et al., 1991; Laudanna et al., 1994). In addition, neutrophil attachment and rolling to cytokine-stimulated vascular endothelium can promote translocation of secretory vesicles to the plasma membrane, thus providing the neutrophil surface with adhesion receptors, including the $\alpha_M\beta_2$ integrin (Carlos and Harlan, 1994; Borregaard et al., 1994).

Granulopoiesis and subsets of neutrophil granules

Neutrophil granules are formed during different stages of maturation of myeloid cells in the bone marrow. Granule formation (granulopoiesis) is a result of a homotypic fusion between identical immature transport vesicles that bud off from the Golgi apparatus, a process that begins in early promyelocytes (Bainton and Farquhar, 1966; Bainton et al., 1971). Proteins of different granule subsets are synthesized at different stages of maturation of neutrophil precursors and proteins of the same subset of granules are produced simultaneously (Borregaard et al., 1995; Le Cabec et al., 1996) (Figure 4). Several transcription factors are involved in controlling granule protein expression in neutrophils, including GATA-1, with a site found in the genes of the α_M subunit of $\alpha_M\beta_2$ integrin and lactoferrin, and c-Myb, with potential sites in the genes of elastase, myeloperoxidase, proteinase-3, and azurocidin (Borregaard and Cowland, 1997). Protein expression and granule formation defects have been observed in acute myeloid leukemia cells, where the normal cell differentiation program is disrupted. Neutrophil granules show great differences in size, density, protein content, as well as tendency for extracellular secretion.

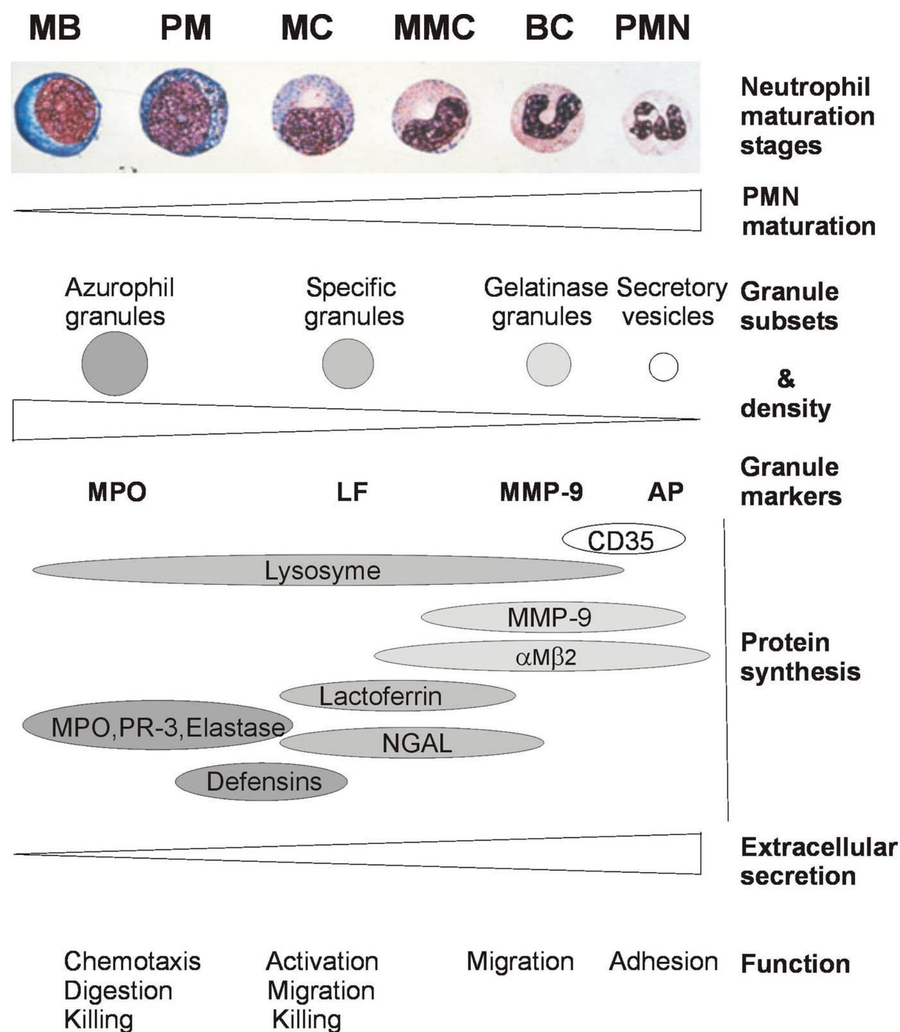


Figure 4. Biosynthetic windows of granules and granule proteins. MB, myeloblast; PM, promyelocyte; MC, myelocyte; MM, metamyelocyte; BC, band cell; PMN, polymorphonuclear neutrophil. Granule proteins: MPO,

myeloperoxidase; PR-3, proteinase 3; LF, lactoferrin, AP, alkaline phosphatase; NGAL, neutrophil gelatinase-associated lipocalin (Modified from Faurschou and Borregaard, 2003).

To date, four types of granules have been detected in neutrophils: the "primary or azurophilic granules", "secondary or specific" granules, "tertiary or gelatinase" granules, and "secretory" vesicles, defined by their content in myeloperoxidase (MPO), lactoferrin (LF), gelatinase B (MMP-9), and latent alkaline phosphatase, respectively. Azurophilic granules appear in the promyelocytic phase, whereas the rest of the granule subsets, in the myelocytic or later stages (Figure 4). Neutrophil degranulation results in the release of granule-containing MMPs which are thought to facilitate neutrophil transmigration through the vascular basement membrane (Delclaux et al., 1996). Both *in vitro* and *in vivo* data describe secretory vesicles as the first (fastest) granules to be released, followed by gelatinase granules, and last, the specific granules (Sengelov et al., 1993). Gelatinase granules can be released in the presence of intracellular Ca^{2+}, at levels above 50 nM plus ionomycin; specific granules, at levels as high as 1μM; and azurophilic granules, only in the presence of extreme values (Nüsse et al., 1998) (see Table 4). Neutrophil granule subsets undergo partial exocytosis once they are in contact with ECM components, thus releasing matrix-degrading enzymes, collagenases and serine proteases to facilitate neutrophil migration.

Exocytosis of both specific and azurophilic granules can also be achieved via disruption of cytoskeleton contacts with cytochalasin B, whereas stimulation of various plasma membrane receptors, such as integrins results in the release of the majority of secretory vesicles (Sengelov et al., 1993, Nüsse et al., 1998). Rho GTPases, including Rac1, Rac2, and Cdc42 have been suggested to play an important role in the regulation of primary granule exocytosis in neutrophils. Studies from knockout mice also suggested that Vav proteins can promote β_2 integrin-association to Rho GTPases and regulate G protein-coupled receptor-induced signaling events which are essential for leukocyte adhesion and phagocytosis (Gakidis et al., 2004).

When neutrophils encounter with bacteria, they activate antimicrobial systems by the release of granule components to the phagocytic vacuole or extracellularly (Joiner et al., 1989). Cytoplasmic granules are discharged in a targeted and regulated manner, a mechanism that enables transformation of neutrophils from passive circulating cells to potent effector cells of the innate immunity. The granule components target bacteria by different ways: (1) disruption of their membrane (defensins, BPI, lactoferrin, and lysosyme); (2) interference with their iron-dependent metabolic pathway (NGAL and lactoferrin); (3) generation of oxygen species (MPO and cytochrome b_{558}); and (4) by induction of chemotaxis of CD4+ and CD8+ T lymphocytes (defensins, azurocidin, and hCAP-18) (reviewed in Faurschou and Borregaard, 2003).

GRANULES /VESICLES	MEMBRANE	MATRIX
Azurophilic granules	CD63 (granulophysin), CD68 (macrophage associated antigen), Stomatin, Presenilin 1, Vascular-type H+-ATPase	Cathepsins, Elastase, Proteinase 3, Defensins, Sialidase, Azurocidin, Lysosyme, Ubiquitin-protein, BPI, MPO, Acid β-glycerophosphatase, α-Mannosidase, β-glucuronidase, Acid mucopolysaccharide, α_1-antitrypsin, N-acetyl-β-glucosaminidase, β-glycerophosphatase, phospholipase A_2
Specific granules	BAP31, $\alpha_M\beta_2$, uPAR, fMLP-R, Thrombospondin-R, Laminin-R, Vitronectin-R, Fibronectin-R, TNF-R, Cytochrome b_{558}, Rap-1, Rap-2, MT6-MMP (MMP-25), Stomatin, CD15 antigens, NB1 antigen, CD15, CD66, CD67, VAMP-2, SCAMP, 19-kD/155-kD proteins, SNAP-23/-25, G-protein$_\alpha$-subunit	Glutaminase, MRP-14, MMP-9, uPA, MMP-8, Lactoferrin, NGAL, CRISP-3, Heparanase, Histaminase, Sialidase Histaminase, Lysosyme, β_2-microglobulin, hCAP-18, Vitamin B12-binding protein, Transcobalamin-I, phospholipase A_2
Gelatinase granules	$\alpha_M\beta_2$, $\alpha_X\beta_2$, uPAR, fMLP-R, Vascular-type H+-ATPase, SCAMP, Cytochrome b_{558}, MT6-MMP (MMP-25), NRAMP-1, VAMP-2, SNAP-23/-25, Diacylglycerol-deacylating enzyme	MMP-9, MRP-14, uPA, Lysosyme, β_2-microglobulin, CRISP-3, Acetyltransferase
Secretory vesicles	$\alpha_M\beta_2$, Alkaline phosphatase, Cytochrome b_{558}, MT6-MMP, fMLP-R, uPAR, C1q-R, Vascular-type H+-ATPase, CD10, CD13, CD14, CD16, CD35, CD45, DAF, SCAMP, VAMP-2	Azurocidin, MRP-14, Plasma proteins (tetranectin, latent alkaline phosphatase, etc.)
Other Compartments		
MVB	$\alpha_M\beta_2$ Alkaline phosphatase Cytochrome b_{558}	M6P-glycoproteins
MLC	LAMP-2/LAMP-1 CI-M6P receptor LAMP-2/LAMP-1	M6P-glycoproteins
PM (markers)	HLA-1, L-selectin	

Table 4. Granule- and secretory vesicle-content of resting neutrophils. Other compartments are also mentioned. MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; BPI, Bactericidal/permeability-increasing protein; MPO, myeloperoxidase; CRISP, cystein-rich secretory protein; HBP, heparin-binding protein; MVB, multivesicular bodies; MLC, multilaminar compartments, LAMP, lysosome-associated membrane proteins; M6P, mannose-6-phosphate, HLA, human leukocyte antigen; hCAP, human cathelicidin protein-18; uPAR, urokinase-type plasminogen activator receptor; NGAL, neutrophil gelatinase-associated lipocalin; DAF, decay-accelarating protein; NRAMP-1, natural resistance-associated macrophage protein-1; SCAMP, secretory carrier membrane protein; SNAP, synaptosome-associated protein, MRP, myeloid-related protein-14; PM, plasma membrane (Table modified from Borregaard and Cowland, 1997).

INTEGRINS AND THEIR LIGANDS

Adhesion receptors, later called integrins were first described in the mid 1980's (Patarroyo et al., 1985a,b). In 1986, the term "integrin" was first designated to describe a protein complex that was involved in the transmembrane linkage between the ECM (fibronectin) and the cytoskeleton (actin) (Tamkun et al., 1986). Soon after, other homologous and structurally related proteins were discovered, thus forming a family of cell surface receptors. Integrins have been detected in all metazoans, including sponges and cnidaria, and organisms as diverse as nematodes and flies (Hynes and Zhao, 2000). In vertebrates, integrins play important roles in certain cell-cell adhesions and in the activation of various signaling pathways. However, no homologs of integrins are present in prokaryotes, plants, or fungi (Whittaker and Hynes, 2002). Integrins are major heterodimeric receptors which are involved in many cell-cell and cell-ECM interactions (reviewed in Hynes, 2002). They are type I transmembrane glycoproteins present on the surfaces of various cells, consisting of two subunits designated α and β that are noncovalently linked to each other. They have a large extracellular domain and a single transmembrane domain, followed by a relatively short cytoplasmic domain (Tuckwell and Humphries, 1993). In mammals, 18 α and 8 β subunits assemble to produce at least 24 distinct heterodimers identified to date, each of which is capable of interacting specifically with membrane-bound, ECM, or soluble protein ligands (reviewed by Hemler, 1990). Figure 2 depicts the complete list of the integrin receptor family with all the possible α- and β-subunit associations.

The integrin family is divided into four major subgroups, based on ligand specificity and cellular expression: β_1 (CD29) integrins (or very late antigens (VLA)), β_2 (CD18) integrins (or leukocyte-specific integrins), β_3 (CD61) integrins (or cytoadhesins), and β_7 (Springer, 1990; Gahmberg et al., 1997; Harris et al., 2000). Integrins bind to their ligands in a divalent-cation-dependent manner (reviewed in Kanazashi et al., 1997; Plow et al., 2000).

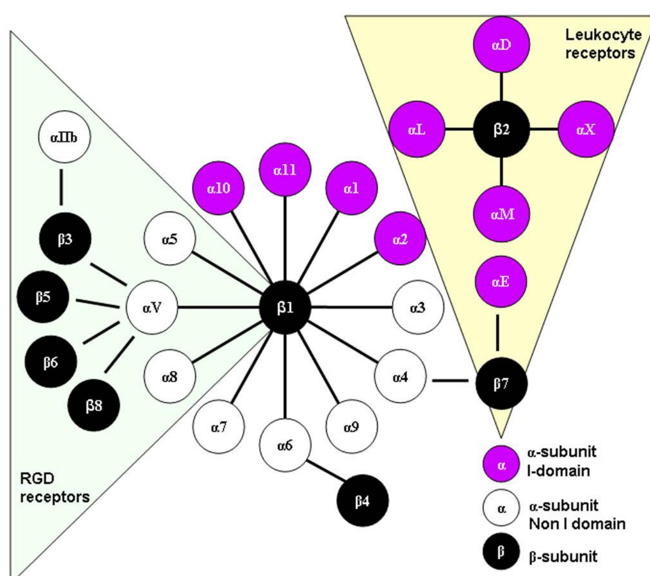


Figure 2. Schematic picture of the integrin family. 8 β subunits can associate with 18 α subunits to form 24 distinct integrin heterodimers, of which 9 out of 18 have an I (inserted) domain. The integrin α-subunits that lack or contain an I domain are shown as white and purple circles, respectively. The β-subunits are shown in black. Integrins that recognize RGD-motif containing ligands are depicted in a green triangle. The leukocyte-specific integrins are within a yellow triangle. Figure modified from Hynes, 2002.

The **β_1 integrins** comprise a large family of receptors which are involved in mediating cell adhesion to ECM proteins, such as collagen, laminin, vitronectin, and fibronectin. They are expressed in almost all types of cells, where they perform multiple functions (Hemler, 1990; Tuckwell and Humphries, 1993; Hynes, 2002). The various combinations of different α (α_1-α_{11}) subunits and a common β_1 subunit increase the diversity of the integrin receptors. Among these integrin heterodimers, $\alpha_1\beta_1$ (VLA-1), $\alpha_2\beta_1$ (VLA-2), $\alpha_3\beta_1$ (VLA-3), $\alpha_4\beta_1$ (VLA-4), $\alpha_5\beta_1$ (VLA-5), $\alpha_6\beta_1$ (VLA-6) are expressed in leukocytes (reviewed in Hemler, 1990). One important example is $\alpha_4\beta_1$ which has been shown to be involved in tethering, rolling, firm adhesion, and transendothelial migration of leukocytes across the endothelium by interacting with components of the ECM, such as fibronectin, and the vascular cell adhesion molecule-1 (VCAM-1, CD106) that is present on the surface of endothelial cells (Elices et al., 1990; Adams and Lobb, 1999). In addition, $\alpha_4\beta_1$ has been reported to mediate homophilic interactions with $\alpha_4\beta_7$ (Altevogt et al., 1995), an integrin which is known to recognize several molecules, including MadCAM-1, VCAM-1, and fibronectin (Berlin et al., 1993) (see Table 1). Although low levels have been detected in blood circulating leukocytes, β_1 integrin receptors can be rapidly upregulated after leukocyte migration through the vascular endothelium. Unlike β_2 integrins, additional signaling is required for upregulation of β_1-integrin expression. This can be achieved by signaling that is generated either by chemotactic molecules or by engagement of β_2 integrins (for example, by antibody-induced cross-linking of β_2 integrins) (Werr et al., 2000a). Furthermore, induction of β_1-integrin expression on the surface of neutrophils strongly correlates with neutrophil transendothelial migration in *in vitro* (Roussel and Gingras, 1997) and *in vivo* (Werr et al., 1998; Werr et al., 2000b) models that mimic neutrophil extravasation.

The **β_3 integrin** family includes the ubiquitous $\alpha_V\beta_3$ integrin, which is a receptor recognizing many ECM components, and $\alpha_{IIb}\beta_3$, the major platelet integrin. $\alpha_V\beta_3$ is found mainly in non-hematopoietic cells, whereas $\alpha_{IIb}\beta_3$ is enriched in platelets. Previous studies confirmed the importance of β_3 integrins in leukocyte adhesion via binding to PECAM-1 (CD31) (Piali et al., 1995), as well as in activation and migration of these cells across endothelial cells and epithelial monolayers (Lawson and Maxfield, 1995; Brown, 1990; Rainger et al., 1999), possibly via binding to the integrin-associated protein, IAP or CD47 (Lindberg et al., 1993). IAP was first isolated as a complex with both $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ integrins (Brown and Frazier, 2001), as well as with $\alpha_V\beta_5$ and $\alpha_2\beta_1$. Recently, IAP and the $\alpha_V\beta_3$ integrin were also shown to bind thrombospondin (TSP-1) via its RGD motif, thus increasing both $\alpha_V\beta_3$-mediated spreading of human melanoma cells and $\alpha_2\beta_1$-mediated chemotaxis of smooth muscle cells (Wang and Frazier, 1998).

The subfamily of integrins that mediate leukocyte firm adhesion to the endothelium, includes four members named either β_2 integrins or according to the cluster of differentiation antigen nomenclature, CD11/CD18 integrins. These leukocyte specific integrins include: $\alpha_L\beta_2$ (LFA-1, CD11a/CD18), $\alpha_M\beta_2$ (Mac-1, CD11b/CD18, CR3), $\alpha_X\beta_2$ (p150,95, CD11c/CD18), and $\alpha_D\beta_2$ (CD11d/CD18). The β_2 integrins are also known to promote interactions of leukocytes with endothelial cells (during firm adhesion and

transmigration), with other leukocytes, as well as with cell-surface bound opsonins on invading bacteria and rejected or hypoxic tissues (Arnaout et al., 1983; Hogg, 1989; Arnaout, 1990a; Springer, 1990; Gahmberg et al., 1990).

The **β_2 integrins** were first purified in the late 1970's from mouse macrophages or human monocytes and lymphocytes (Milstein et al., 1979; Davignon et al., 1981), which affected several lymphocyte functions, but they were not known to be adhesion proteins. Convincing proof for their adhesive nature was obtained by inducing adhesion using phorbol esters followed by inhibition with antibodies recognising the β_2-chain (Patarroyo et al., 1995a,b; Rothlein and Springer, 1986). Since the recognition of the integrin receptor family over the past 20 years (Hynes, 1987), enormous progress has been done in elucidating the integrin structure and function. Currently, integrins are the best-understood family of cell adhesion receptors.

The $\alpha_L\beta_2$ integrin is primarily expressed in lymphocytes but also found in all other leukocytes. It was first described on murine and human lymphocytes by using monoclonal antibodies (mAbs) that could inhibit both cytotoxic T cell-mediated killing and T cell proliferation (Davignon et al., 1981; Sanchez-Madrid et al., 1982). Later, $\alpha_L\beta_2$ was shown to play an essential role in leukocyte adhesion and migration across the endothelium by its ability to bind to several intracellular adhesion molecules (ICAMs), especially ICAM-1 which is present on the surface of endothelial cells, to E-selectin, and to collagen type I (Kotovuori et al., 1993; Garnotel et al., 1995; Gahmberg, 1997). Initiation of an immune response requires the formation of the immunological synapse between T cells and antigen-presenting cells (APCs). This process involves the association of $\alpha_L\beta_2$ integrin with ICAM-1 (Bachmann et al., 1997), ICAM-3 (Bleijs et al., 2000), and other adhesion molecules, aligning the plasma membranes of the two cells in proximity to each other (Grakoui et al., 1999). Recent reports point out the importance of $\alpha_L\beta_2$ in organ transplant and treatment of autoimmune diseases, since mAbs directed against it substantially increased graft survival in several animal models (Poston et al., 2000; Nicolls et al., 2002), and impaired the symptoms of psoriasis in clinical trials (Gottlieb and Bos, 2002).

The $\alpha_M\beta_2$ integrin is expressed on cells of the myeloid lineage, such as granulocytes, monocytes, and macrophages, and it is capable of mediating many of the proinflammatory functions in these cells (Dana et al., 1991). It binds to a broad spectrum of ligands, such as membrane-anchored ICAMs (ICAM-1, -2, and –4) (reviewed in Gahmberg et al, 1997; Gahmberg, 1997), and to several soluble ligands, including the complement fragment iC3b, fibrinogen, factor X, heparin, E-selectin (Crutchfield et al., 2000), bacterial lipopolysaccharide (LPS) (Wright and Jong, 1986), urokinase-type plasminogen activator receptor (uPAR) (Pluskota et al., 2003), catalase (Davis, 1992), myeloperoxidase (Johansson et al., 1997), junctional adhesion molecule 3 (JAM-3), and proteinases, such as proteinase 3, cathepsin G, neutrophil elastase (Cai and Wright, 1996), various ECM proteins (Yakubenko et al., 2002 and references therein) (see Table 1).

ADHESION MOLECULES	LIGANDS	LOCALIZATION
Integrins		
β_2 integrins		
$\alpha_L\beta_2$ (LFA-1)	ICAM1-5, LRP	Lymphocytes
$\alpha_M\beta_2$ (Mac-1)	ICAMs, E-selectin, iC3b, FG, Factor X, heparin, uPAR, Thy-1, LRP, FN, NIF, JAM-C, NE, GPIbα, Cyr61	Granulocytes/monocytes
$\alpha_X\beta_2$ (p150,95)	ICAM-1, iC3b, FN, FG, CD23	Macrophages
$\alpha_D\beta_2$	ICAM-1/-3, VCAM-1	Macrophages
β_7 integrins		
$\alpha_4\beta_7$	VCAM-1, MAdCAM-1, FN	Lymphocytes (lymph nodes)
$\alpha_E\beta_7$	E-cadherin	Lymphocytes (intraepithelia)
β_1 integrins		
$\alpha_1\beta_1$ (VLA-1)	Collagens, Laminin-1	Lymphocytes
$\alpha_2\beta_1$ (VLA-2)	Collagens, Laminin-1, MMP-1	Lymphocytes
$\alpha_3\beta_1$ (VLA-3)	Laminins, TSP	Lymphocytes
$\alpha_4\beta_1$ (VLA-4)	VCAM-1, FN, OP, TSP	Lymphocytes
$\alpha_5\beta_1$ (VLA-5)	FN, TSP, ADAMs, endostatin	Lymphocytes
$\alpha_{6/7}\beta_1$ (VLA-6/-7)	Laminins	Lymphocytes
$\alpha_8\beta_1$ (VLA-8)	FN, VN, OP, TN-C, NN, LAP	Mesangial/myofibroblast
$\alpha_9\beta_1$ (VLA-9)	VCAM-1, TN-C, OP	Neutrophils
$\alpha_{10}\beta_1$ (VLA-10)	Collagen type II	Chondrocytes
$\alpha_{11}\beta_1$ (VLA-11)	Collagen type I	Chondrocytes
β_3 integrins		
$\alpha_V\beta_3$	CD31, FN, VN, TSP, vWF, TN-C, OP, thrombin, agrin, fibrillin, canstatin, tum, MMP-2, ADAMs, BSP, Thy1	Macrophages
β_5 integrins		
$\alpha_V\beta_5$	VN, OP, HIV tat, BSP, LAP, canstatin	Endothelial cells
Selectins		
L-selectin (CD62L)	E-/-P selectins, GlyCAM-1, CD14, MAdCAM-1, CD34, sLeX, PSGL-1	All leukocytes
E-selectin (CD62E)	ESL-1, sLeX, PSGL-1, L-selectin, CLA, SSEA-1	Endothelial cells
P-selectin (CD62P)	PSGL-1, sLeX, CD24	Endothelial/platelets
Members of IgSF		
ICAM-1 (CD54)	$\alpha_{L/M/X}\beta_2$, MMP-9	Endothelium/monocytes
ICAM-2 (CD102)	$\alpha_{L/M}\beta_2$	Endothelium/leukocytes
ICAM-3 (CD50)	$\alpha_{L/D}\beta_2$	Endothelium/leukocytes
ICAM-4	$\alpha_{L/M/X}\beta_2$, $\alpha_4\beta_1$, $\alpha_V\beta_{1,3,5}$, $\alpha_{IIb}\beta_3$	Erythrocytes
ICAM-5	$\alpha_L\beta_2$	Neurons
VCAM-1 (CD106)	$\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_D\beta_2$	Endothelial cells
PECAM-1 (CD31)	PECAM-1, $\alpha_V\beta_3$	Endothelium/leukocytes
MAdCAM-1	$\alpha_4\beta_7$, L-selectin, $\alpha_4\beta_1$	Endothelium (intestine)

Table 1. Molecules involved in adhesive interactions between leukocytes and the vascular endothelium. IgSF, immunoglobulin superfamily; TSP, thrombospondin; OP, osteopontin; TN, tenascin; NE, neutrophil elastase; NN, nephronectin; LAP, TGFβ latency associated protein, iC3b, inactivated complement component 3b; can, canstatin; tum, tumstatin; BSP, bone sialic protein, LRP, LDL-related protein; NIF, neutrophil inhibitory factor; JAM-C, junctional adhesive molecule-C, GPIbα, glycoprotein Ibα; Thy1, thymus cell antigen 1.

MAbs raised against the $\alpha_M\beta_2$ integrin dramatically alleviated the degree of ischemia-reperfusion injury in several animal models of phagocyte-dependent acute tissue injury (Vedder et al., 1988). $\alpha_M\beta_2$-integrin antibodies also inhibited the accumulation of phagocytes in damaged tissues and their interaction with the complement iC3b, thus preventing development of insulin-diabetes mellitus in susceptible mouse strains (Hutchings et al., 1990). Antibody treatment was also found to be successful in models of experimental autoimmune encephalomyelitis (Huitinga et al., 1993) and colitis (Palmen et al., 1995) by inhibiting the accumulation of neutrophils and monocytes to the inflammatory sites. Finally, anti-α_M mAb therapy led to strong attenuation of the severity of disease in two models of arthritis (Taylor et al., 1996; de Fougerolles et al., 2000), and showed reduced injury in several ischemia-reperfusion models by blocking leukocyte-endothelial cell interactions (Cornejo et al., 1997).

The $\alpha_X\beta_2$ integrin is mainly expressed on tissue macrophages, and is a marker for hairy cell leukemia. It is also expressed, at lower levels, on dendritic cells, granulocytes, natural killer (NK) cells, lymphoid cells lines and populations of activated T and B cells (Cabanas, 1999). $\alpha_X\beta_2$ binds to ICAM-1, iC3b, fibrinogen, and type I collagen (Garnotel et al., 2000). The most recently discovered β_2 integrin, $\alpha_D\beta_2$ is primarily found on monocytes, macrophages, oesinophils and other leukocytes, and mediates binding to ICAM-3 and VCAM-1 (van der Vieren, 1995). This interaction may contribute to the homing and keeping leukocytes in certain tissues.

Structure and function of leukocyte β_2 integrins

The structural characteristics and functional roles of leukocyte β_2 integrins have been extensively reviewed recently (Gahmberg et al., 1997; Arnaout, 2002; Shimaoka et al., 2002; Takagi and Springer, 2002). The β_2 integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, and $\alpha_D\beta_2$) consist of α- (1063, 1137, 1144, and 1084 residues, respectively) and β- (747 residues) subunits (Figure 3A). The extracellular domains of all β_2-integrin subunits contain several potential N-glycosylation sites: 12 in α_L, 19 in α_M, 8 in α_X, 11 in α_D, and 6 in the β-chain (reviewed in Gahmberg et al., 1997) and the structures of the oligosaccharides have been determined (Asada et al., 1991). Divalent cations are essential for integrin functions by regulating the integrin structure in a state in which they increase or suppress binding to physiological ligands (reviewed in Plow et al., 2000). To date, the primary structures of all four β_2 integrin α- and β-subunits have been described by molecular cloning (Corbi et al., 1987; Arnaout, 1988; van der Vieren et al., 1995).

Sequence analysis of the α-subunits showed approximately 60-65 % homology between the α_M (170 kDa), α_X (150 kDa), and α_D (155 kDa) subunits, and about 35 % of homology to α_L (180 kDa). The α_L, α_M, α_X, and α_D subunits are encoded by three distinct genes that are all clustered on chromosome 16 (Marlin et al., 1986; Arnaout et al., 1988). The β_2 gene is on chromosome 21 (Suomalainen et al., 1986; Marlin et al., 1986). Each integrin α-subunit contains seven, 60- amino-acid long, homologous segments in the amino-terminal region, and with resemblance to a domain present in the trimeric G protein β-subunit, which are

predicted to fold into a seven-bladed β-propeller domain (Tuckwell et al., 1994; Springer, 1997). Along with the predicted I-like domain (βA) from the β-subunit, they both interact to form the "head" of the integrin (Figure 3B). Each α-subunit contains three characteristic EF-hand-like divalent cation-binding sites within the β-propeller sheets 5-7, with resemblance to metal-binding motifs DXXDXXXD present in several calcium-binding proteins, including calmodulin and parvalbumin (Arnaout, 1990b).

Half of all integrin α-subunits contain an additional, 200-amino-acid long, I domain which is inserted between the propeller β-sheets 2 and 3 (Arnaout, 1988; Michishita et al., 1993; Diamond et al., 1993a; Springer, 1997), and is homologous to a plasma glycoprotein von Willebrand factor (Colombatti and Bonaldo, 1991). The three-dimensional architecture of the extracellular domains of the integrin α- and β-subunits has been revealed by crystallization, electron microscopy, and nuclear magnetic resonance (NMR) (Xiong et al., 2001, 2002; Beglova et al., 2002; Takagi et al., 2002). Based on the crystal structure of the extracellular domains of $\alpha_V\beta_3$, it has been predicted that the I domain lies on top of the β-propeller domain (Springer, 1997) (Figure 3C).

The I domain plays an essential role in ligand binding (Diamond et al., 1993a; Michishita et al., 1993; Colombatti et al., 1993), with a partial contribution from the β-propeller (Stanley et al., 1994; Dickeson et al., 1997). Also, the EF-hand-like repeats indirectly participate in ligand binding (Xiong et al., 2001). This conclusion is strongly supported by mAb-mapping, mutation, and I-domain deletion studies (Diamond et al., 1993; Randi and Hogg, 1994; Leitinger and Hogg, 2000a). The crystal structures of α_L (Qu and Leahy, 1995), α_M (Lee et al., 1995), α_2 (Emsley et al., 1997; 2000), and α_1 (Salminen et al., 1999) I domains have been solved. They all adopt a classical dinucleotide-binding (Rossmann) fold, with five parallel and one antiparallel β-strand in the center, surrounded by seven α-helices, and a divalent cation-binding site, referred to as the Metal Ion-Dependent Adhesion Site (MIDAS) at the apex of the I domain. The binding of a divalent cation, such as Mg^{+2} or Mn^{+2}, is coordinated by oxygen-containing side chains from five amino acids of the I domain and a predicted sixth residue provided by the ligand (Lee et al., 1995; Emsley et al., 2000). The metal cations and MIDAS ion, located at the bottom of the β-propeller and in the I domain of the α-subunit, respectively, are reported to affect the stability of the integrin's structure. For example, the MIDAS cation in the I domain increases the integrin's resistance to thermal or chemical denaturation and has the ability to directly activate integrins (reviewed in Xiong et al., 2003). Mutational studies have shown the importance of these divalent cations in ligand binding (Kamata et al., 1995a; McGuire and Bajt, 1995). Indeed, inhibition of the integrin function is achieved by EDTA, which chelates divalent cations (Altieri, 1991).

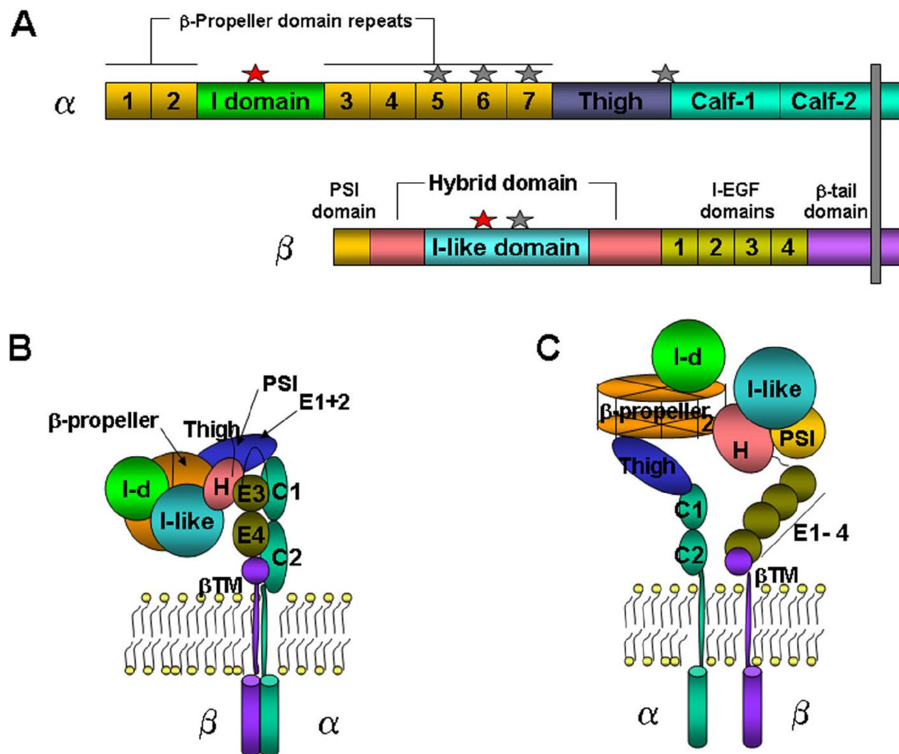


Figure 3. Schematic structure of the leukocyte integrin. (**A**) represents the integrin's primary structure, including divalent cation-binding sites (Mg^{+2} as red and Ca^{+2} as grey stars). (**B** and **C**) are schematic representations of the bent (inactive) and strainghtened (active) conformations of the integrin, respectively. The arrangement of domains is based on the three-dimensional crystal structure of $\alpha_V\beta_3$ integrin, with an I domain added between the 2nd and 3rd β-propeller repeats. Each domain is coloured as in **A**. I-d, I-domain; I-EGF, integrin-epidermal growth factor domain; PSI, plexin/semaphorin/integrin; β-TM, β-tail domain.

The common β_2-subunit of leukocyte integrins is different from their corresponding α-subunits, but is homologous to β_1 and β_7 by 46 %. An interesting feature of the primary sequence of the β-subunit is a carboxy-terminal cysteine-rich repeat made of four EGF-like domains which lie below the hybrid domain (Beglova et al., 2002). Another cysteine-rich region (seven cysteine residues) is located within the 54-amino -acid long PSI domain (Bork et al., 1999) that lies amino-terminal to the hybrid domain, and named after its sequence homology with plexins, semaphorins, and integrins. All together, 56 cysteine residues are present in the β-subunit. These cysteine-rich regions are known to keep the integrin in its inactive conformation (Zang and Springer, 2001). A sequence with a homology to the I-domain of the α-subunit is also found in the amino-terminus of the integrin β-subunit and denoted the I-like domain. The I-like domain is 241 residues long and contains a MIDAS motif (DXSXS), similar to that of the I-domain. Based on mutational studies, the I-like domain (via a residue at position 243) is important in forming contacts with the α-subunit β-propeller (via a residue at position 438) (Zang et al., 2000; Xiong et al., 2001) and with various ligands in integrins which lack I domains (Goodman and Bajt, 1996). These contacts are essential for proper folding of the integrin subunits (Huang et al., 1997; Huang and Springer, 1997). Additional contacts are known to be formed between the α- and β-subunits: for example, (1) between the hybrid domains and the β-propeller, (2)

between the EGF3 and calf-1 domains, (3) between the EGF-4 and calf-2, and (4) between the β-subunit transmembrane domain (βTM) and calf-2 (reviewed in Arnaout, 2002).

Loss of heteromerization of the integrin during biosynthesis caused by mutations in the gene encoding the β-subunit resulted in reduced β_2-integrin cell-surface expression and function on leukocytes, leading to a rare human inherited disease, called leukocyte adhesion deficiency-I (LAD-I) (Anderson and Springer, 1987; Arnaout, 1990a; Hogg and Bates, 2000). Expression of nonfunctional β_2 integrins was also observed in LAD-I patients carrying mutations in the MIDAS motif of the I-like domain in the β-subunit (Hogg et al., 1999). Polymorphonuclear neutrophils (PMNs) and monocytes from LAD-I patients fail to migrate through the vascular endothelium or become fully activated because of lack of adherence, actin cytoskeleton rearrangement, and spreading on ICAM-1- or ECM-coated surfaces (Shappell et al., 1990). This explains why LAD-I patients are exposed to life-threatening bacterial infections. The same phenotype was observed in β_2-integrin knockout mice (Scharffetter-Kochanek et al., 1998). In accordance to these results, a study on the contribution of each subunit separately in adhesion or migration of cells showed that α-subunit-expressing cells mediated adhesion and spreading on a variety of integrin ligands, but failed to support cell migration. However, cells expressing only the β_2-subunit showed a migratory phenotype and successfully attached on a subset of integrin ligands but failed to spread on these ligands (Solovjov et al., 2005). In the I-domain-containing integrins, ligand binding appears to be indirectly regulated by the I-like domain. High resolution electron microscopic (EM) studies suggested that head separation of the integrin α- and β-subunits was not triggered by ligand binding (Weisel et al., 1992; Du et al., 1993; Erb et al., 1997; Takagi et al., 2002), and did not result in high affinity ligand binding by integrins (Luo et al., 2003). However, recent studies provide evidence that loss of heterodimerization between the integrin TM domains increase ligand binding affinity, whereas integrin valency or clustering remain unchanged (Luo et al., 2005).

The cytoplasmic tails of integrins are smaller in size (< 50 residues) than their extracellular domains and are pivotal in regulating ligand binding and signaling function (Woodside et al., 2001). All α-chains contain a conserved GFFKR motif proximal to the cell membrane (Williams et al., 1994). Truncation of either one of the integrin tails can lead to a constitutively active receptor (O'Toole et al., 1994). Unlike β_2 integrins which have a NPXF motif in their β-chain, the cytoplasmic domains of the rest of the integrin family contain two conserved NPXY motifs (van Kooyk et al., 1998). Mutations of the threonine residues in a conserved motif, SXXTT which is present in β_1, β_2, and β_7 integrins (reviewed by Ylänne, 1998), decreased leukocyte adhesion via inhibiting complex formation between $\alpha_L\beta_2$ integrin and ICAM-1 (Hibbs et al., 1991a, 1991b; Williams et al., 1994). These motifs may be important, not only in signaling, but also in integrin endocytosis and localization (van Kooyk et al., 1998) (see below). Several reports support the idea that association of the membrane proximal regions of the α and β subunit cytoplasmic domains is needed to keep the integrin in its low affinity state (Hughes et al., 1996; Vallar et al., 1999; Lu et al., 2001a; Takagi et al., 2001; Vinogradova et al., 2002). Eventually, disruption of the interacting sites between these two tails leads to an active integrin.

Structural features of the integrin I-domains alone or in association with their binding partners, ICAMs have been recently documented (reviewed in Springer and Wang, 2004). Three distinct conformations of the I domain have been reported, denoted closed, intermediate, and open, which reflect the low, intermediate, and high affinity state of the integrin for ligand binding, respectively (Shimaoka et al., 2003). The first crystal structure of α_L I domain in a complex with ICAM-1 was determined at 3.3-Å resolution (Shimaoka et al., 2003). This structure revealed the open ligand binding conformation of the I domain, bearing a Mg^{+2} in its MIDAS site which directly coordinates a glutamic acid (Glu-34) residue in ICAM-1.

Integrin-Ligand interactions	**Integrin and Ligand binding sites**	**Method**	**References**
$\alpha_L\beta_2$-ICAM-1	MIDAS-D1 (Glu34)	X-ray	Shimaoka et al., 2003
$\alpha_L\beta_2$-ICAM-2	MIDAS-D1 (Glu37)	Mutagenesis	Casasnovas et al., 1999
$\alpha_L\beta_2$-ICAM-3	MIDAS-D1 (Glu37)	X-ray	Song et al., 2005
$\alpha_L\beta_2$-ICAM-4	I-domain-D1 (W19,77,93 ; L80; R97)	Mutagenesis	Hermand et al., 2004
$\alpha_M\beta_2$-ICAM-4	I-domain-D1 (W19,77,93 ;L80;R97) -D2(E151;T154)	Mutagenesis	Hermand et al., 2004
$\alpha_{IIb}\beta_3$-ICAM-4	unknown -D1 (Q30,36;G3 2;K33;W77) & D2 (E151)	Mutagenesis	Hermand et al., 2004
$\alpha_V\beta_3$-ICAM-4	unknown -D1 (R52,97;Y6 9;D73;L80;K33;W66,77) -D2 (E151;T154)	Mutagenesis	Hermand et al., 2004
$\alpha_V\beta_{1/5}$-ICAM-4	unknown -D1 (W19,66;F1 8;V20;R92,97;A94;T94,S96) -D2 (K118)	Mutagenesis	Mankelow et al., 2004
$\alpha_M\beta_2/\alpha_X\beta_2$-FG	I-domain-P1 (γ400-411) & P2 (γ377-395) sites	Mutagenesis	Ugarova & Yakubenko, 2001
$\alpha_V\beta_3$-FN	I-like domain- FNIII D10 (RGD)	X-ray EM	Xiong et al., 2002 Adair et al., 2005
$\alpha_V\beta_3$-ADAM-15	I-domain-RGD	Mutagenesis	Zhang et al., 1998
$\alpha_{IIb}\beta_3$-FN	β-propeller - FNIII D9-10 (RGD)	Mutagenesis	Kauf et al., 2001 Xiao et al., 2004
$\alpha_{IIb}\beta_3$-FG	β-propeller/I-like domain- C-terminal (γ400-411)	Mutagenesis	Kamata et al., 2001 Xiao et al., 2004
$\alpha_1\beta_1/\alpha_2\beta_1$-Laminin	α-subunits-α_2 chain short arm	Blocking Abs	Colognato et al., 1997
$\alpha_2\beta_1$-collagen	MIDAS- G(F/L/M)OG**E(131)**R	X-ray	Emsley et al., 2000
$\alpha_4\beta_1$-FN	$\alpha_4\beta_1$-FN14 (P**RARI**)	Mutagenesis	Sharma et al., 1999
$\alpha_4\beta_1$-FN	β_1 chain (I**D(130)**S)- CS1 (EI**LDV**PST)	Blocking peptides	Guan and Hynes, 1990
$\alpha_4\beta_1$-VCAM-1	β_1 chain (I**D(130)**S)- D1 (C-D loop)	X-ray Mutagenesis	Kamata et al., 1995b
$\alpha_5\beta_1$-FN	β-propeller/I-like domain- FN7-10 (RGD)	EM	Takagi et al., 2003
$\alpha_E\beta_7$-E-Cadherin	MIDAS-Glu31	Mutagenesis	Higgins et al., 2000

Table 2. Integrin-ligand interaction sites. ICAM, intercellular adhesion molecule; D1, domain 1; Abs, antibodies; FN, fibronectin; FG, fibrinogen; RGD, Arg-Gly-Asp; VCAM, vascular cell adhesion molecule. Important amino acids for binding are marked in bold.

More recently, another crystal structure of α_L I domain in complex with ICAM-3 (Glu-37) has been determined at a resolution as high as 1.65-Å (Song et al., 2005). These structures allow us to conclude that the binding of ICAMs onto the I domains of integrins is mediated by a common docking mechanism. Other techniques utilized to characterise the association between leukocyte integrins and their ligands, include EM, NMR, mAb mapping, and mutagenesis of either ligand or receptor. Several other integrin complexes and their precise mechanism of association have been determined (see Table 2).

Activation of leukocyte β_2 integrins

The term ”activation” applied to integrins describes changes that are required in order to increase ligand binding affinity, whereas activation of signaling receptors describes changes that result in enhanced signal transduction mediated through ligand binding (reviewed in Calderwood, 2004). Four levels of integrin signaling have been theoretically described: “inside-out”, “outside-in”, “anchorage”, in which integrins anchor to the cytoskeleton; and “clustering”, in which integrins become clustered to stabilize adhesion.

Intensive mutagenesis and EM studies have revealed that both integrin activation and signaling are mediated by conformational chances which occur bi-directionally, from the cytoplasmic domains to the headpiece of the integrin (a process termed “inside-out”) and vice-versa (a process termed “outside-in”) (reviewed in Lub et al., 1995; Liddington and Ginsberg, 2002; Shimaoka et al., 2002). These two processes play an important role during cell proliferation (van Seventer et al., 1990) or in prevention of cell apoptosis (Koopman et al., 1994).

A great number of cell surface receptors and integrin-associated proteins are known to be involved in signaling events which are important in regulating the integrin affinity. The “**inside-out**” signals can be initiated via stimulation of other cell surface receptors, including the tyrosine kinase-coupled T cell receptors (TCR) or G protein-coupled chemokine receptors (Dustin and Springer, 1989; Lollo et al., 1993; Constantin et al., 2000) and CD44 (Vermot-Desroches et al., 1995). Signals mediated by these receptors are thought to modulate $\alpha_L\beta_2$ integrin cytoplasmic tail-mediated triggering of enhanced adhesiveness in the extracellular domain (O’Rourke et al., 1998; van Kooyk and Figdor, 1993). Increased integrin adhesiveness by inside-out signals allows circulating leukocytes to strongly attach to the endothelium or to interact with antigen-presenting cells (APCs; Springer, 1995; Grakoui et al., 1999). However, the mechanism involved in inducing a high affinity state of $\alpha_L\beta_2$ has remained poorly understood. Cross-talk of β_2 integrins with ICAM-derived peptides (Li et al., 1993, 1995), ICAMs (Bleijs et al., 2000), other integrins (Imhof et al., 1997; Chan et al., 2000), selectins (Ruchaud-Sparagano et al., 2000) selectin ligands (Evangelista et al., 1999), PECAM-1 (Piali et al., 1993), and other cell surface molecules (Petty and Todd, 1996; Porter and Hogg, 1998) can occur to modulate integrin function. For example, there is cross-talk between β_2 integrins and several other membrane-associated proteins, including $\alpha_4\beta_1$ integrin, urokinase plasminogen activator receptor (uPAR), IAP, and members of the tetraspan protein family (reviewed in Worthylake and Burridge, 2001). For example, the ECM protein, TSP-1 binds to IAP and $\alpha_v\beta_3$ integrin through two different binding sites,

resulting in increased intracellular signaling mediated via the $\alpha_v\beta_3$. This event has been shown to be important in T cell adherence and activation (Reinhold et al., 1999), demonstrating that association of integral membrane proteins *in cis* with integrins can have a strong effect on intracellular signaling. To date, several other associations between integrins and other cell-surface receptors have been identified (Table 3). Also, engagement of $\alpha_4\beta_1$ integrin or L-selectin has been reported to induce adhesiveness of both $\alpha_L\beta_2$ and $\alpha_M\beta_2$ integrins for ICAM or activated endothelial cells, an event mediated by increases in actin polymerisation, as well as by integrin/selectin clustering (Simon et al., 1999; Chan et al., 2000).

Activation by signals within the cells leads either to an active conformation (extended conformation, high affinity state) or clustering (high avidity state) of the integrin, both necessary for increased ligand binding (reviewed in Zell et al., 1999). It has been reported that association between the α- and β-cytoplasmic domains restricts the integrin in its inactive (bent conformation, low affinity state; Figure 3B) conformation (Takagi et al., 2002). Dissociation of these domains, mediated by intracellular signals, induced a switchblade-like opening of the integrin extracellular domains to an extended conformation (Figure 3C) (Takagi et al., 2001; Vinogradova et al., 2002). Mutagenesis (Lu and Springer, 1997) and fluorescence resonance energy transfer (FRET) (Kim et al., 2003) studies also provides evidence for conformational changes occuring in the cytoplasmic tails during physiological activation. The extended conformation describes an open conformation of the headpiece which corresponds to the high affinity state of the integrin as demonstrated by NMR (Beglova et al., 2002) and EM studies (Takagi et al., 2002).
The cytoplasmic regions of $\alpha_L\beta_2$ have been reported to modulate cell adhesion. Truncation of the β_2 cytoplasmic domain or mutations performed in that region (T758TT/AAA), both abolished adhesion of COS cells to ICAM-1 (Hibbs et al., 1991a). The TTT motif can be phosphorylated (Fagerholm et al., 2004) and mutations of the motif affects adhesion, actin reorganization, and cell spreading (Peter and O'Toole, 1995). Conformational changes in $\alpha_L\beta_2$ are also thought to be induced after association of the integrin cytoplasmic tails with talin, or adaptor proteins, such as cytohesin-1 (Nagel et al., 1998; Hmama et al., 1999; Geiger et al., 2000). Talin head is known to bind to the β-chain cytoplasmic domain, thus triggering the separation of the two cytoplasmic domains (Calderwood and Ginsberg, 2003, Vinogradova et al., 2004). Also, recruitment of cytohesin-1 to the plasma membrane and its association with $\alpha_L\beta_2$ increases adhesion to ICAM-1 (Kolanus et al., 1996). Recent data demonstrate that cytohesin-1, phosphatidylinositol 3(-OH) kinase (PI(3)K), and Rap-1 are directly involved in chemokine-mediated $\alpha_L\beta_2$ lateral mobility in lymphocytes (Constantin et al., 2000; Shimonaka et al., 2003). Rap-1 is a potent inside-out signal. Rap-1, PI(3)K, and PKC are all reported to be involved in the activation of $\alpha_L\beta_2$ (Katagiri et al., 2000).

Monoclonal antibodies recognizing the ligand-binding site of $\alpha_L\beta_2$ (Petruzelli et al., 1995; Bazzoni and Hemler, 1998) and $\alpha_M\beta_2$ (Diamond and Springer, 1993) have been reported to bind preferentially to activated integrins, to ligand-occupied forms of integrins, or induce activation themselves through conformational changes in the extracellular domains to a high affinity for ligand. A 10,000-fold increase in

affinity of $\alpha_L\beta_2$ for its ligand, ICAM-1 could be obtained by creating an engineered conformational change in the integrin I domain (Shimaoka et al., 2001; Lu et al., 2001b).

PROTEIN	INTEGRIN	REFERENCES
Tetraspanins		
TM4SF	β_1 & β_2 integrins, $\alpha_{IIb}\beta_3$	Porter and Hogg 1998; Dedhar, 1999; Tarrant et al., 2003
Growth-factor receptors		
EGF-R	$\alpha_V\beta_3$, $\alpha_2\beta_1$	Moro et al., 1998;Yu et al., 2000
PDGF-R-β	$\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$	Borges et al., 2000
Insulin-R	$\alpha_V\beta_3$	Schneller et al., 1997; Maile et al., 2002
ErbB2	$\alpha_6\beta_1$, $\alpha_6\beta_4$	Falcioni et al., 1997; Gambaletta et al., 2000
VEGF-R-2	$\alpha_V\beta_3$, $\alpha_V\beta_5$	Borges et al., 2000; Reynolds et al., 2002
GPI-linked receptors		
uPAR (CD87)	$\alpha_{L/M/X}\beta_2$, $\beta_{1,3,5}$	Kindzelskii et al., 1997; Carriero et al., 1999; Yu et al., 2000; Simon et al., 2000
FcγRIIIB (CD16B)	$\alpha_M\beta_2$	Petty and Todd, 1996
CD14	$\alpha_M\beta_2$	Petty and Todd, 1996
Thy-1 (CD90)	$\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_V\beta_3$	Wetzel et al., 2004; Choi et al., 2005; Saalbach et al., 2005
Ig superfamily proteins		
CD147 (EMMPRIN)	$\alpha_3\beta_1$, $\alpha_6\beta_1$	Berditchevski et al., 1997
CD47 (IAP)	$\alpha_2\beta_1$, $\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$	Brown et al., 1990; Wang and Frazier, 1998
CD46 (MCP)	$\alpha_3\beta_1$	Lozahic et al., 2000
CD36	$\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$	Dorahy et al., 1996; Thorne et al., 2000
P2Y heptaspanin	$\alpha_V\beta_3$	Erb et al., 2001
Syndecan-4	$\alpha_5\beta_1$	Mostafavi-Pour et al., 2003
Transporters/Ion channels		
16K V-ATPase	β_1 integrins	Skinner and Wildeman, 1999
Kv1.3	β_1 integrins	Levite et al., 2001
GIRK	β_1 integrins	McPhee et al., 1998; Ivanina et al., 2000
BAP31	$\alpha_M\beta_2$	Zen et al., 2004
ICln	$\alpha_{IIb}\beta_3$	Larkin et al., 2004

Table 3. **Integrins that interact with transmembrane proteins.** TM4SF, transmembrane-4 superfamily; EGF-R, epidermal growth factor receptor; PDGF-R, platelet-derived growth factor receptor; Thy1, thymus cell antigen 1; EMMPRIN, extracellular matrix MMP inducer; IAP, integrin associated protein; MCP, membrane cofactor protein; P2Y, purinergic receptor; Kv1.3, a voltage-gated potassium channel; GIRK, G protein-coupled inwardly rectifying potassium channel; BAP31, B cell receptor-associated protein; ICln, a chloride channel regulatory protein.

Lateral redistribution and clustering of integrins (avidity regulation) may also have an impact on cellular adhesion even though the affinity for ligand remains unchanged (Takada et al., 1997). For example, treatment of leukocytes with phorbol esters induces clustering of β_2-integrins by 10-fold (integrin avidity) without altering affinity (Kucik et al., 1996), thus inducing β_2-integrin-dependent leukocyte adhesion. Phorbol esters directly activate PKC and bypass early signaling events which are transmitted by other receptors (Wright and Silverstein, 1982). $\alpha_L\beta_2$ clustering on the cell surface can also be induced by interactions with cytoskeletal proteins (Stewart et al., 1998; van Kooyk et al., 1999). Treatment of

lymphocytes with high concentrations of cytochalasin-D, which disrupts actin filaments, inhibited cell adhesion to coated ICAM-1 induced by CD3 antibodies or phorbol esters (Stewart et al., 1998).

Transmission of signals into the cell by integrins can be generated after interactions with ligands or counter-receptors on other cells or soluble ligands (the process is referred to as "**outside-in**" signaling). One of the earliest events in β_2 integrin signaling is the activation of both protein tyrosine kinases (PTKs), including members of the focal adhesion kinase (FAK) family ($p125^{FAK}$ and Pyk2) and the Src-kinase family (Fgr, Hck, Lyn, and Syk), which in turn can trigger downstream activation of multiple intracellular signaling cascades, known to regulate cell morphology, cell growth and survival, adhesion, and migration. Various cytoskeleton-associated proteins including paxillin, tensin, cortactin, and talin become tyrosine phosphorylated in PMNs after ligand binding to β_2 integrins. This strongly correlates with integrin β_2-mediated PMN spreading since such an effect was efficiently blocked by tyrosine kinase inhibitors (reviewed in Berton and Lowell, 1999). Integrin engagement results in tyrosine phosphorylation of other cytoskeletal-associated proteins, such as the cytoplasmic tyrosine kinase Fgr (Berton et al., 1994), the Syk tyrosine kinase (Lin et al., 1995), phospholipase Cγ2 (Hellberg et al., 1996), Vav (Zheng et al., 1996), the proto-oncogene product Cbl (Ojaniemi et al., 1997), the mitogen-activated protein kinases ERK1/ERK2 (McGilvray et al., 1997), $p125^{FAK}$ (Fernandez et al., 1997), and ZAP-70 (Soede et al., 1999). β_2 integrin-dependent PMN adhesion/spreading on fibrinogen correlates with Fgr or Lyn kinase activation (Berton et al., 1994; Yan et al., 1995). These findings are also well-supported by data showing that leukocyte adhesion deficiency (LAD-I) PMNs are unable to activate Fgr. In addition, both β_2 integrin-deficient LAD-I cells (Berton et al., 1994) and leukocytes expressing $\alpha_L\beta_2$ integrin which lacks the I domain show impaired "outside-in" signaling (Leitinger and Hogg, 2000). Furthermore, double knockout mice for Hck and Fgr (*hck*$^{-/-}$ *fgr*$^{-/-}$) showed impaired PMN spreading on fibrinogen or ICAM-1 and failed to release large amounts of ROIs, even after treatment with TNF or fMLP. Double mutant macrophages showed similar integrin-mediated cell spreading defects (Lowell et al., 1996). Interestingly, collagen type I-binding to $\alpha_L\beta_2$ integrin results in tyrosine phosphorylation of both the α- and β-subunit of the integrin (Garnotel et al., 1995). However, not much is known on the precise mechanisms by which these tyrosine kinases become enzymatically activated by integrins *in vivo*.

Previous reports supported the idea that the $\alpha_L\beta_2$ integrin prevails in inactive state (bent conformation) when expressed on circulating lymphocytes (Carman and Springer, 2003; Salas et al., 2004) and that it becomes activated by endothelium-anchored chemokines through specific G protein-coupled receptors (GPCRs) which are present on the surface of leukocytes (Constantin et al., 2000). In this case, integrins are known to regulate adhesion merely by clustering as lymphocytes move along the vessel wall. Recently, Ronen Alon and colleagues proposed that chemokine-mediated $\alpha_L\beta_2$ activation in T cells occurs "locally", with an immediate lymphocyte arrest and "bidirectionally", which involves instantaneous inside-out as well as outside-in conformational arrangements (extension of bent $\alpha_L\beta_2$) of individual integrin molecules (Shamri et al., 2005).

MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a family of structurally related and highly conserved zinc-dependent endopeptidases collectively capable of degrading most components of the basement membrane and ECM (Birkedal-Hansen et al., 1993; Nagase and Woessner, 1999). To date, there are at least 25 secreted or membrane-bound known human MMPs (Sternlicht and Werb, 2001; Visse and Nagase, 2003). Data generated from intensive studies on MMP activities in different cells and tissues, as well as studies from knockout animals witness the importance of these enzymes in many normal physiological processes (e.g. embryonic development, bone resorption, angiogenesis, and wound healing) and pathological processes (rheumatoid arthritis, multiple sclerosis, periodontal disease, and tumor growth and metastasis) (Murphy and Hembry, 1992; Nagase and Woessner, 1999; Egeblad and Werb, 2002; Hamano et al., 2003).

CLASSIFICATION

Most human MMPs can be divided according to their sequence homology, substrate specificity, and cellular location (Shingleton et al., 1996) into several subclasses: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and others (Murphy et al., 1991a; Nagase, 1994; Pendas et al., 1997) (Figure 5).

STRUCTURAL FEATURES

The basic multidomain structure of MMPs comprises: (1) an amino-terminal domain; (2) a catalytic domain; and (3) a carboxy-terminal domain. The amino-terminal domain contains a 17-29 amino acid *signal peptide*, used as a signal for secretion of the proenzyme into the ER and eventually out of the cell, and a 77-87 residue propeptide domain or *pro-domain*, that is responsible for keeping the enzyme inactive until proteinase activity is needed. The pro-domain contains a highly conserved sequence, PRCG(V/N)PD in which the cysteine residue interacts with the catalytic zinc atom in the active site, thus maintaining the proMMP in its latent form. Disruption of the Cys-zinc covalent bond by a water-zinc interaction (called the cysteine switch) and removal of the pro-domain may lead to a conformational change of the enzyme from inactive to catalytically active form (van Wart and Birkedal-Hansen, 1990; Springman et al., 1990).

The *catalytic domain* contains about 160-180 residues, including calcium ion-binding sites and the consensus zinc-binding HEBXHXBGBXHS motif (H for Histidine; E for glutamic acid; B for bulky hydrophobic amino acid; G for Glycine; X for variable amino acid; S for Serine) (Murphy et al., 1991b; Puente et al., 1996; Pendas et al., 1997). However, MMP-11 has threonine in place of serine (Stocker et al., 1995) and MMP-17 has valine in place of serine (Puente et al., 1996). Together with the catalytic zinc ion, this motif is essential for the proteolytic activity of MMPs. Additionally, there is a second, structural zinc and at least one calcium ion located near the catalytic zinc, both important for stabilizing the MMP structure (Borkakoti, 2000).

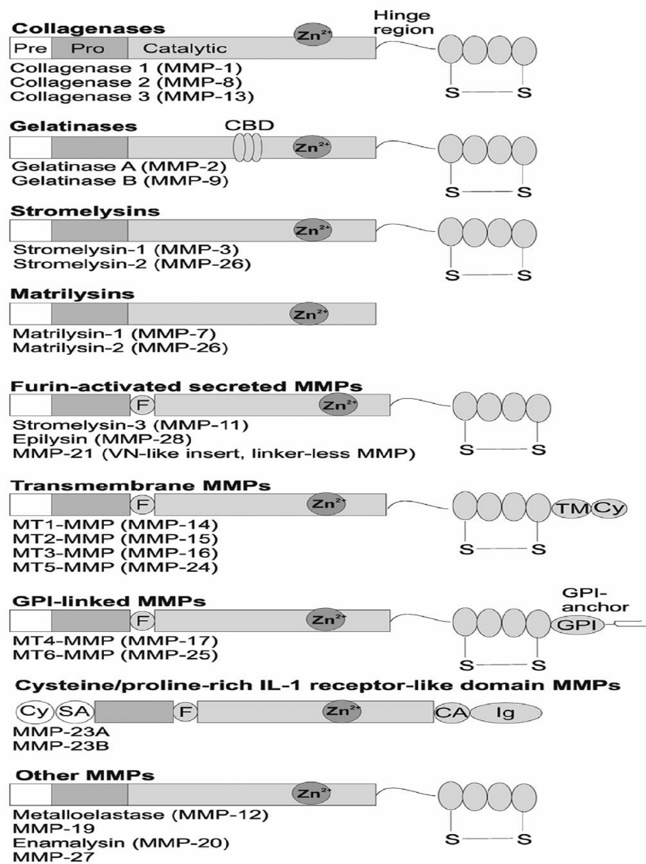


Figure 5. Domain structures of human MMPs. Pre, signal sequence; Pro, propeptide with cysteine switch sequence; Zn, zinc-ion binding site; CBD, collagen/gelatin binding domain or fibrinectin-type II repeats; F, furin cleavage site; VN, vitronectin-like insert; TM, transmembrane domain; Cy, cytoplasmic tail; SA, signal anchor; GPI, GPI-anchoring domain; CA, cysteine array; Ig, Ig-like domain. Modified from Westermarck and Kähäri, 1999.

The catalytic domain is linked to the *hemopexin* domain by a 5-50 residue proline-rich *hinge region*. In comparison to MMP-7 (matrilysin-1), MMP-23, and MMP-26 (matrilysin-2), all other MMPs contain a ~200 residue and highly conserved C-terminal domain with a sequence similarity to a serum protein named hemopexin. The hemopexin domain contains four repeats that are connected by a disulfide bridge at both ends, thus allowing the domain to fold into a four-bladed propeller structure, with a calcium ion in the middle of a central disc like structure (Morgunova et al., 1999). This domain is known to play a functional role in protein in substrate binding, in interactions with the natural tissue inhibitors of metalloproteinases or TIMPs (van Wart and Birkedal-Hansen, 1990; Sanchez-Lopez et al., 1993; Baragi et al., 1994), and in forming MMP homodimers (Cha et al., 2002). Recently, the hemopexin domain of MMP-2 was shown to be important for the activation of the protease (Morgunova et al., 1999; Overall et al., 1999). Additional functional domains are present in some of the other MMP members. For example, three repeats of the fibronectin-type II domain are present in the catalytic domain of gelatinases, MMP-2 and MMP-9 that are capable of mediating enzyme binding to gelatin substrates (Murphy et al., 1994).

A *transmembrane* domain is known to be present in all MT-MMPs after an extension beyond the hemopexin domain that localizes these proteases into the cell membrane. Unlike MT-MMPs, MMP-19 contains a further extension of the hemopexin domain but lacks the transmembrane-spanning region.

REGULATION OF MMPs

The expression, secretion, and activity of MMPs in normal tissues are subject to tight control. Most MMPs are expressed at very low levels but their expression and activation can be rapidly induced at times of tissue remodeling, such as during wound healing (Nagase and Woessner, 1999).

Transcriptional regulation

Transcription of MMPs is regulated by many factors, including cytokines, growth factors, oncogenic cell transformation, physical stress, and cell-cell and cell-ECM interactions. Also, hormones, oncogenes, and chemical agents like phorbol esters can upregulate (for example, IL-1, IL-6, TNF-α, EGF, PDGF, bFGF, NGF, PMA) or downregulate (for example, IFN-γ, TGF-β, retinoids, and glucocorticoids) the expression of MMPs (Birkedal-Hansen et al., 1993; Borden and Heller, 1997; Westermarck and Kähäri, 1999; Nagase and Woessner, 1999; Sternlinct and Werb, 2001). Unlike other MMPs, TGF-β does not suppress but slightly induces transcription of MMP-2 (Brown et al., 1990; Overall et al., 1991). Many of these inducers of MMP expression are known to act at the level of transcriptional activation of the gene. The promoters of many MMP genes (such as MMP-1, -3, -7, -10, -12, -13, and -19) include an activator protein-1 (AP-1) consensus element which is located approximately at position -70 and one or two copies of the polyomavirus enhancer A-binding protein-3 (PEA-3) element at position between -140 and -200. AP-1 and PEA-3 sites interact with the Fos and Jun families and the Ets family of transcription factors, respectively (Westermarck and Kähäri, 1999). Several reports have shown that these *cis*-elements are important for the regulation of MMP gene expression at the basal gene-expression level and in response to extracellular stimuli like growth factors, cytokines, and phorbol esters (Angel et al., 1987; Westermarck and Kähäri, 1999). For example, the MAPK pathway, which comprises ERK1/2, JNK, and p38 proteins can induce the expression of AP-1 transcription factors, which is considered by many to be the major activators of MMP-1, -3, -9, and -13 expresion (Westermark and Kähäri, 1999). However, several reports show differences between promoter regions of MMP-2 and other MMPs (Matrisian, 1994). MMP-2 gene is distinguished by the lack of the AP-1 and PEA-3 regulatory elements in the promoter region, which explains the lack of transcriptional regulation of MMP-2 expression. The MMP-2 promoter also lacks the TATA site which is important in regulating the transcription of most promoters, as well as the upstream TGF-β inhibitory element (TIE) and AP-1 element (Matrisian, 1994; Huhtala et al., 1990; Tryggvason et al., 1990). The latter is shown to interact with AP-1 transcription complexes. Also, single-nucleotide polymorphisms have been identified within several MMP gene promoters, which affect the rate of transcription, and influence the development and progression of several diseases, including cancer (Ye, 2000). Finally, the expression of many MMPs can be upregulated by ligand and/or antibody-binding to integrins (Werb et al., 1989; Dumin et al., 2001). For example, $\alpha_L\beta_2$-, $\alpha_M\beta_2$- and $\alpha_3\beta_1$-integrin ligation promotes the expression of MMP-9. Accordingly, inhibition of these integrins by antisense oligonucleotides leads to reduced MMP-9 expression (Aoudjit et al., 1998; DiPersio et al., 2000).

Zymogen activation

Interaction between the unpaired cysteine residue which is located in the prodomain and the conserved zinc atom at the active site is thought to be responsible in maintaining the latency of MMPs (Springman et al., 1990; van Wart and Birkedal-Hansen, 1990). Disruption of the covalent bond which associates the zinc atom with the cysteine residue and coordination with H_2O can lead to the cleavage of the prodomain and further activation of proMMPs (Nagase and Woessner, 1999; Curran and Murray, 2000). Most MMPs are known to be secreted as inactive zymogens and activated extracellularly by many different mechanisms (Will et al., 1996; Cao et al., 1996; Kinoshita et al., 1998; Mazzieri et al., 1997; Wang et al., 2000). Most proMMPs are also known to be activated in the pericellular space by serum or tissue proteinases, bacterial proteinases, and other MMPs. Proteinases, such as trypsin, plasmin, kallikrein, chymase and mast cell chymase, are capable of activating proMMPs *in vitro*. ProMMPs can also be activated by various non-proteolytic agents, including mercurial compounds (aminophenyl mercuric acetate or APMA), SH-reactive agents, chaotropic agents, reactive oxygen, detergents, and heat treatment (Springman et al., 1990; Saarialho-kere et al., 1992; Murphy and Knäuper, 1997; Nagase and Woessner, 1999). In case of MMP-2, -8, and -9, activation can occur when exposed to acidic pH followed by neutralization (Davis, 1991).

In vivo proMMP activation is found to localize in both to the intracellular and extracellular milieus, and on the cell surface (Nagase and Woessner, 1999; Visse and Nagase, 2003). MMP-11 (stromelysin-3), MMP-21, -23, -28, and MT-MMPs are activated intracellularly by Golgi-associated, furin-like serine proteases (Pei and Weiss, 1996; Kang et al., 2002; Visse and Nagase, 2003). It has been reported that in the case of MMP-23, a single cleavage is enough to induce both activation and release of MMP-23 from the cell surface (Pei et al., 2000).

MMP-2 is resistant to proteolytic activation by serine proteases and other endopeptidases because it lacks a basic amino acid motif, present in most other MMPs (van Wart and Birkedal-Hansen, 1990; Curran et al., 2000; Vihinen and Kähäri, 2002). However, MMP-2 activity is highly regulated by MT-MMPs, membrane localized MMPs which are important activators of proMMPs in the pericellular space (Brinckerhoff et al., 2000). Firstly, MT-MMPs, such as MT1-MMP and MT3-MMP form cell-surface complexes with tissue inhibitors of MMPs, TIMP-2 and TIMP-2 or TIMP-3, respectively, through their C-terminal tail (Strongin et al., 1995; Zhao et al., 2004). Secondly, TIMP-free MT-MMP can activate proMMP-2 (Zucker et al., 1998; Wang et al., 2000; Overall et al., 2000). In many cases, MMP activation is achieved by autocatalytical activation, the cell-surface associated urokinase-type plasminogen activator (uPA)/plasmin-dependent activation system, and by other proteinases (Carmeliet et al., 1997; Mazzieri et al., 1997).

Inhibition of enzymatic activity

Specific inhibition of the activity of MMPs can be achieved in the pericellular milieu by the non-specific, circulating protease inhibitors, α_2-macroglobulin (α_2M), serpins, and by the specific TIMPs. α_2M is a homotetramer, made of two pairs of non-covalently associated dimers, capable of inhibiting MMP activity in the synovial fluid, serum, and other body fluids. It can also inhibit the activity of several other enzymes, including serine-, cysteine-, and aspartate proteinases. Serpins, including α_1-antitrypsin (α_1-proteinase inhibitor) and plasminogen activator inhibitor (PAI)-1 and -2, are glycoproteins of 50-100 kDa, involved in regulating the proteolytic activity of MMPs in various tissues (Saarialho-Kere et al, 1992; Birkedal-Hansen et al., 1993).

TIMPs are known to be the key endogenous regulators of MMP activities in tissues. Four known members of the TIMP family (TIMPs-1, -2, -3, and -4) have been identified to date, all anchored in the ECM or secreted extracellularly (for reviews see Gomez et al., 1997; Westermarck and Kähäri, 1999; Brew et al., 2000). In spite of the fact that all TIMPs share very similar structural features and inhibitory capabilities against almost all MMPs, they exhibit differences in tissue distribution, in their ability to interact with the latent forms of MMPs and to inhibit their activity, in solubility, and in their gene regulation patterns (Nagase and Woessner, 1999; McCawley and Matrisian, 2000; Sternlinct and Werb, 2001). They contain 12 conserved cysteine residues required for building up six disulfide bonds, which keep the two domains in a rigid conformation (Douglas et al., 1997; Gomez et al., 1997). TIMPs form non-covalent 1:1 stoichiometric complexes with the zinc-binding catalytic site of active MMPs, thus inhibiting proteolytic activity (Gomis-Rüth et al., 1997). The N-terminal domain of TIMPs contains a consensus sequence VIRAK which is required for MMP inhibition, whereas the C-terminal domain seems to affect their specificity (Murphy et al., 1991b; DeClerck et al., 1993; Goldberg et al., 1992; Ogata et al., 1995; Bigg et al., 1997). TIMPs are capable of inhibiting not only active MMPs but also can prevent autocatalytic activation of several MMPs (DeClerck et al., 1991; Strongin et al., 1993) by forming complexes with proenzymes.

In vitro TIMPs can inhibit the activation of almost all MMPs. For instance, TIMP-1 and TIMP-2 can preferentially inhibit MMP activation by forming a complex with proMMP-9 and proMMP-2, respectively, via the hemopexin domain. TIMP-1 blocks the activity of most MMPs, with the exception of MT1- and MT3-MMPs. Finally, TIMP-1 inhibits cleavage and secretion of heparin-binding EGF-like growth factor and its receptor HER-2 from the surface of breast cancer cells (Dethlefsen et al., 1998; Codony-Servat et al., 1999). Whereas for TIMP-1 to be expressed in culture requires growth factors, cytokines, and phorbol esters, the expression of TIMP-2 is constitutive (Gomez et al., 1997). TIMP-2 is expressed in culture by a variety of cells in a constitutive manner and also inhibits the activity of most MMPs (Strongin et al., 1993), but not MMP-9. This particular MMP inhibitor is capable of blocking the shedding of TNF-α receptors I and II (TNF-αRI and II). Unlike the other TIMPs, which are secreted in soluble forms in tissues and body fluids, TIMP-3 is insoluble and found associated with the ECM (Leco et al., 1994). TIMP-3 can inhibit the shedding of pro-TNF-α, L-selectin, and IL-6 receptor from the surface of human peripheral leukocytes and myeloma

cells (Borland et al., 1999). TIMP-4 is mainly expressed in the adult human heart (Greene et al., 1996) and can also form a complex with proMMP-2 (Goldberg et al., 1989).

Finally, several proteinases from the ADAM family can also be inhibited by TIMPs due to their structural similarities with MMPs in the active site. For example, both TIMP-1 and TIMP-3 can inhibit ADAM10 and ADAMTS4 (aggrecanase-1) (Amour et al., 2000; Kashiwagi et al., 2001). TIMP-3 alone also inhibits ADAM17 (TNF-a convertase) and ADAMTS5 (aggrecanase-2) (Kashiwagi et al., 2001).

ROLE OF INTEGRINS AND MMPs IN LEUKOCYTE MIGRATION

REGULATION OF CELL ADHESION AND MIGRATION

Leukocyte migration is a complex process, controlled by a wide spectrum of adhesion molecules that are expressed on both leukocytes and endothelial cells, and by the presence of chemotactic molecules. These molecules, as well as growth factors, are responsible for the establishement of a polarised cell migration and there is enough evidence to prove that signaling from both phospholipids and proteins from the Rho family of small GTPases are also involved in directed cell motility (Rickert et al., 2000). Migration of leukocytes is essential for immune responses, tissue repair, and embryonic development. When cell migration is not regulated, this may lead to cancer invasion and metastasis.

Integrins have attracted much attention by their strong contribution to the anchorage of cells to the ECM and migration across it. During spreading of cells on the ECM, integrins form large aggregates with their other associated proteins at sites where the plasma membrane and the matrix remain in close contact. These are called cell-matrix adhesion sites. Engagement of integrins by ECM proteins induces binding of filamentous (F)-actin to the integrin cytoplasmic domains, mediated by several adaptor proteins of the cytoskeleton (Geiger et al., 2001). In addition, different structures described by small adhesions that are present in membrane protrusions, elongated adhesions made of an assembly of fibronectin molecules, and large adhesions formed by F-actin stress fibers, occur in spreading cells and represent the “focal complexes”, “fibrillar adhesions”, and “large adhesions”, respectively (Zamir and Geiger, 2001). In fibroblasts, structures where integrins and signaling molecules are associated intracellularly are described as “focal adhesions”. Many integrins bind to fibronectin via the same integrin-binding motif, Arg-Gly-Asp (RGD), also present in many other ECM proteins. For example, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, both expressed in leukocytes, have been shown to bind to fibronectin but via a different mechanism (Weber et al., 1996). $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_L\beta_2$, and $\alpha_M\beta_2$ integrins are involved in the regulation of chemokine-induced adhesion and spreading of leukocytes (Peled et al., 2000; Voermans et al., 2000). Mice with a deletion of the α_4-subunit gene showed decrease in T-cell homing to Peyer patches (Arrojo et al., 1996). Antibodies against the same subunit blocked SDF-1-induced transendothelial migration of CD34+ cells (Peled et al., 2000). $\alpha_2\beta_1$ integrin also plays a prominant role in leukocyte migration and inhibition of this integrin prevented neutrophil migration by 70 % (Werr et al.,

1998; 2000b). Data from mice that lack β_2 integrins showed impaired neutrophil infiltration to inflammatory sites (Borjesson et al., 2003). Moreover, antibody cross-linking of β_2 integrins has been shown to upregulate β_1-integrin expression (Werr et al., 2000a). Similar effect on β_1-integrin expression was detected in leukocytes after transendothelial migration (Kubes et al., 1995; Werr et al., 1998; 2000b). Taken together, adhesion and migration of neutrophils is strongly associated with β_1 and β_2 integrin expression and activation.

During migration, signaling through the chemokine receptors (inside-out) triggers changes in the distribution of filamentous F-actin in leukocytes, which undergo morphological changes, characterized by a switch from a spherical to a polarized shape (Howard and Oresajo, 1985). During cell polarization, leukocyte integrins, chemoattractant receptors and other adhesion molecules, cytoskeletal proteins and intracellular regulatory molecules change their cellular localization. Polarization of leukocytes occurs not only during cell attachment to the vascular endothelium mediated by integrins, but also during antigen presentation and target-cell recognition (reviewed in Sanchez-Madrid and Del Pozo, 1999). A polarized morphology of leukocytes was first described to be similar to that of a migrating amoebae, with a leading edge at the front and a uropod at the rear of a migrating cell (Wilkinson, 1986). T cells recognize and bind to APCs through their leading edge (Kupfer and Singer, 1989). Changes in T cells, such as cell surface protein clustering, cytokine secretion, and cytoskeletal reorganization triggered after contact with APCs have been extensively studied (Kupfer and Singer, 1989).

A number of receptors are concentrated at the leading edge, including $\alpha_V\beta_3$, uPAR, and fMLP-R in neutrophils; CCR2, CCR5, and FAK in T cells; and CXCR4 in B cells, which are able to sense chemotactic gradients, thus guiding leukocytes to migrate in a polarized manner. Antibody-specific cross-linking to L-selectin induced the translocation of intracellular vesicle-stored CXCR4 to specific sites on the surface of T cells, called lipid rafts (Sitrin et al., 2004). At the uropod, several reports show localization of ICAMs, L-selectin, $\alpha_M\beta_2$, PSGL-1, FcγR-IIIb, CD2, CD43, and CD44 (reviewed in Sanchez-Madrid and del Pozo, 1999; Webb et al., 2002), which play a pivotal role in cell adhesion, thus facilitating cell migration. However, the $\alpha_5\beta_1$ integrin can be translocated via endosomes from the uropod to the leading edge of neutrophils (Pierini et al., 2000). Release of the uropod triggers cell migration. Some of these receptors when bound to the substratum, become linked to the actin cytoskeleton during cell migration. Interactions between the cytoskeleton and the cell surface receptors are required for the formation of membrane protrusions, such as lamellipodia (broad, sheet-like structures) and filopodia (thin cylindrical needle-like projections), both structures located at the leading edge (Nobes and Hall, 1995). The ERM proteins (ezrin, radixin, and moesin) are localized at cell-surface protrusions and form a link between the actin cytoskeleton and the cytoplasmic tails of the membrane receptors. It has been suggested that the association of fMLP receptor with talin regulates actin polymerization in neutrophils (Jesaitis et al., 1993).

Changes in the actin cytoskeleton in response to extracellular stimuli are mainly regulated by the Rho family of small GTPases, such as Rac, Cdc42, and RhoA (Nobes and Hall, 1995). Rac1 mediates

lamellipodia formation (Ridley et al., 1992), Cdc42 promotes filopodia formation (Kozma et al., 1995), and RhoA controls focal adhesion formation and actomyocin-mediated contraction in fibroblasts (Ridley and Hall, 1992). Myocin-induced contraction of the actin network can pull the cell to migrate, suggesting that RhoA is implicated in the uropod retraction. Rho is known to be involved in $\alpha_L\beta_2$/ICAM-1-dependent leukocyte aggregation and adhesion through integrins (Laudanna et al., 1996; Sanchez-Madrid and del Pozo, 1999). The actin network is assembled at the leading edge which allows continuous movement of the leukocyte while it disassembles at the rear (Mitchison and Cramer, 1996). Myosin, a motor protein, seems to play an important role in migration of leukocytes and other cells by gathering several adhesion receptors, including integrins towards the uropod, as suggested by studies performed on myosin-deficient amoebae (Wessels et al., 1988). Second messengers (calcium and cAMP) and protein (PKA, PKC, and FAK) or lipid (PI3K) kinases also play important role in cell migration. In migrating leukocytes, calcium is involved in the regulation of the myosin (located at the uropod)-induced cell contraction, which is manifested by the high Ca^{2+} levels observed at the cell rear. Adhesion receptors attached to the substrate move backwards as the cell migrates, while at the same time, renewal of these receptors such as integrins occurs at the leading edge. Altogether, a combination of high calcium and cAMP concentrations, and low Rho GTPases activity can lead to disruption of actin filaments, as well as induction of cell detachment via myosin-dependent contraction and formation of the uropod, whereas low levels of these second messengers and Rho GTPases and high phosphoinositide levels can promote cell adhesions and formation of lamellipodia and filopodia at the leading edge via increase in integrin activation and actin filament assembly (Janmey, 1994). During transmigration, RhoA and its effector, p160ROCK, are both required for retraction of the leukocyte tail. Inhibition of RhoA led to the translocation of β_2 integrins from front to the rear of migrating leukocytes (Worthylake et al., 2001), as well as failure to retract the uropod. Thus, coordination of these molecules at specific places in the cell can determine the assembly or desessembly of the actin cytoskeleton and the localization of membrane protrusions and retractions. Finally, several MMPs, including MT1-MMP and MMP-2 were found to colocalize at membrane protrusions. Interaction of MMPs with their natural inhibitors, TIMPs at these sites might be the key mechanism for the regulation of cell surface-MMP activation and eventually, the control of the invasive phenotype of cells (reviewed in Chen and Wang, 1999). MT1-MMP can promote signal transduction and regulate gene expression. Indeed, transfection of cells with the MT1-MMP gene induced the activation of the extracellular signal-regulated protein kinase (ERK) cascade and Rac 1, leading to increased cell migration (Takino et al., 2004; Cao et al., 2004). MT1-MMP mediated-cleavage of the integrin α_V subunit of the $\alpha_V\beta_3$ integrin resulted in focal adhesion kinase (FAK) phosphorylation and cell migration. Interestingly, shedding of CD44 (Kajita et al., 2001) and LRP (Rozanov et al., 2004) ectodomains by MT1-MMP promotes the release and translocation of the cytoplasmic fragment from the cell surface into the nucleus for transcriptional activation (Nakamura et al., 2004).

CELL SURFACE ASSOCIATION OF MMPs AND OTHER PROTEASES

MMPs are secreted as zymogens from inside the cell to the cell surface and into the extracellular environment where they are able to degrade both ECM and non-ECM proteins. It remains unclear how these enzymes make it to the correct location at the cell surface and how the proteolytic activity is controlled at the pericellular space. However, it has been suggested that MMP-binding to cell surface proteins can have an effect on intracellular signaling, can facilitate proenzyme localization and activation, mediate cell motility by disruption of cell contacts with the ECM and promote internalization of the enzyme. For example, integrins are shown to act as receptors for several proteases, including MMPs. Such interactions have been detected in caveolae, in invadopodia, and at the leading edge of migrating cells, where directed proteolytic activity is needed. The first interaction between an integrin ($\alpha_V\beta_3$) and a MMP (MMP-2) was identified on the surface of melanoma cells and angiogenic blood vessels (Table 5). This complex was shown to be involved in tumor growth and angiogenesis *in vivo* (Brooks et al., 1998). Immunofluorescence and confocal microscopic studies showed the colocalization of MT1-MMP and TIMP-2 with caveolin-1 in the same membrane microdomains with MMP-2 and $\alpha_V\beta_3$ integrin (Puyraimond et al., 2001), termed caveolae. Caveolae are membrane invaginations enriched in cholesterol and glycolipids and serve as sites for clustering of various integrins and proteases (Smart et al., 1999). They are involved in the transport of lipids, in signal transduction pathways and in non-clathrin-mediated endocytosis (reviewed in van Deurs et al., 2003). MT1-MMP was shown to activate $\alpha_V\beta_3$ through proteolytic cleavage, suggesting that coordinated expression and localization of these molecules may be important for cancer cell invasion and metastasis. Furthermore, there is evidence that the $\alpha_V\beta_3$ integrin has modulatory properties on MMP-2 activity by binding to its C-terminal domain (Brooks et al., 1996; Brooks et al., 1998). Inhibition of the $\alpha_V\beta_3$/MMP-2 complex formation by either the MMP-2 C-terminal domain (Pfeifer et al., 2000) or a small molecule inhibitor, TSRI265 (Silletti et al., 2001) dramatically suppressed angiogenesis *in vivo*, demonstrating that this interaction is essential for endothelial cell proliferation and migration. The TSRI265 molecule neither blocked MMP-2 protease activity nor $\alpha_V\beta_3$-mediated cell adhesion. Since then, several other important protease associations with integrins have been reported (Table 5), suggesting that pericellular proteolysis may be activated and targeted by integrins and other cell surface receptors. Another gelatinase, proMMP-9 has been detected on the surface of many cell types, including leukocytes, endothelial cells, keratinocytes, and in a variety of malignant cells as a proenzyme and often free of TIMP-1 (reviewed in Seiki et al., 2003). A complex between proMMP-9 and TIMP-1 was detected in the culture media of breast epithelial MCF10A cells but not on the cell surface after treatment with phorbol esters, suggesting that cell surface-bound proMMP-9 may prevent its inhibitor from binding to it (Toth et al., 1997).

In leukocytes, uPA could bind to its receptor, uPAR, and to $\alpha_M\beta_2$ simultaneously, forming a trimolecular complex where $\alpha_M\beta_2$ could serve as a signaling receptor (Pluskota et al., 2003). This interaction is likely to be mediated by both the kringle and proteolytic domains for uPA and the I-domain for $\alpha_M\beta_2$. A peptide sequence within the α_M subunit and outside the I domain was shown to bind uPAR (Simon et al.,

2000). This complex plays an essential role in the migration of inflammatory cells and vascular homeostasis. The uPA/uPAR complex was also found associated with the $\alpha_5\beta_1$ integrin and capable of promoting adhesion and migration of Chinese hamster ovary cells as well as intracellular signal transduction through the integrin. The uPA/uPAR and uPAR/β_1 integrin complexes were also reported to be present in caveolae of chondrocytes (Schwab et al., 2001; Tarui et al., 2003). Recently, proMMP-9 was found to be associated with ICAM-1 (Fiore et al., 2002) and DNA repair protein Ku (Monferran et al., 2004) on the surface of leukemic cells. ICAM-1 cleavage by MMP-9 resulted in tumor cell resistance to natural killer cell-mediated cytotoxicity.

Soluble proteases	Associated proteins	Cell-surface expression	References
MMPs			
MMP-1	$\alpha_1\beta_1$	myocytes	Stricker et al., 2001
	$\alpha_2\beta_1$	keratinocytes	Dumin et al., 2001
	EMMPRIN	lung carcinoma	Guo et al., 2000
	PAR1	breast carcinoma	Boire et al., 2005
MMP-2	$\alpha_V\beta_3$	melanoma, endothelial	Brooks et al., 1996; 1998
	LRP	fibroblasts	Yang et al., 2001
	collagen chains	fibroblasts	Steffensen et al., 1998
	TSP-2	fibroblasts	Yang et al., 2001
	TIMP-2	malignant cells	Olson et al., 1997
	caveolin-1	endothelial	Puyraimond et al., 2001
	Hsp90α	fibrosarcoma	Eustace et al., 2004
	MT1-MMP	fibrosarcoma	Strongin et al., 1995
	BS	-	Fedarko et al., 2004
MMP-3	osteopontin	-	Fedarko et al., 2004
MMP-7	CD44HSPG	epithelial	Yu & Woessner, 2000
	TM4SF	-	Maecker et al., 1997
	CD151	rectal carcinoma	Shiomi et al., 2005
MMP-9	collagen chains	epithelial/fibrosarcoma	Okada et al., 1992
	RECK	fibrosarcoma	Takahashi et al., 1998
	CD44	melanoma	Yu & Stamenkovic, 1999
	ICAM-1	leukemias	Fiore et al., 2002
	LRP	fibroblasts	Hahn-Dantona et al., 2001
	Ku protein	macrophages/leukemia	Monferran et al., 2004
	TIMP-1	fibroblasts	O'Connell et al., 1994
	TSP-1	malignant cells	Rodriguez-Manzaneque, 2001
	$\alpha_{L/M}\beta_2$	neutrophils/leukemias	Stefanidakis et al., 2004
	$\alpha_5\beta_1$	epithelial	Wang et al., 2003
	$\alpha_3\beta_1$	mammary carcinoma	Morini et al., 2000
	$\alpha_V\beta_5$	fibrosarcoma	Bjorklund et al., 2004
	DMP-1	-	Fedarko et al., 2004
MT1-MMP	$\alpha_V\beta_3$	endothelial	Galvez et al., 2002
	β_1 subunit	endothelial	Galvez et al., 2002
	CD44	fibrosarcoma	Mori et al., 2002
	TIMP-2	breast carcinoma	Imai et al., 1996
	collagen type I	gingival fibroblasts	Tam et al., 2002
	RECK	fibrosarcoma	Oh et al., 2001

Serine proteases			
uPA	uPAR, which in turn interacts	malignant	Ellis et al., 1999
	with : $\alpha_{M/X}\beta_2$	neutrophils	Xue et al., 1994
	: $\alpha_V\beta_3$	fibrosarcoma	Xue et al., 1997
	: $\alpha_V\beta_5$	mammary carcinoma	Carriero et al., 1999
	: $\alpha_3\beta_1$	mammary carcinoma	Wei et al., 2001
elastase	$\alpha_M\beta_2$	neutrophils	Cai and Wright, 1996
seprase	uPAR	melanoma	Artym et al., 2002
	$\alpha_3\beta_1$	melanoma	Monsky et al., 1994
Dipeptidyl peptidase IV	$\alpha_3\beta_1$	fibroblasts	Ghersi et al., 2002
cathepsin G	FPR	leukemias	Sun et al., 2004
	HIV-1 gp120	leukemias	Avril et al., 1994
	membrane Gp	platelets/neutrophils	Molino et al., 1993
proteinase 3	$\alpha_M\beta_2$	neutrophils	David et al., 2003
plasmin	Annexin II	kidney cells	MacLeod et al., 2003
Cysteine proteases			
Cathepsin B	annexin II	tumors	Mai et al., 2000
	α_2-M	bone metastases	Arkona & Wiederander, 1996
ADAMs			
ADAM-2	$\alpha_6\beta_1$	Oocytes	Chen and Sampson, 1999
ADAM-7	$\alpha_4\beta_1$	T cell leukemia	Bridges et al., 2005
	$\alpha_9\beta_1$	T cell leukemia	Bridges et al., 2005
	$\alpha_4\beta_7$	T cell leukemia	Bridges et al., 2005
ADAM-9	$\alpha_6\beta_1$	fibroblasts	Nath et al., 2000
	$\alpha_9\beta_1$	oocytes	Eto et al., 2002
	$\alpha_V\beta_5$	Myeloma	Zhou et al., 2001
ADAM-12	$\alpha_9\beta_1$	haematopoietic	Zhang et al., 1998
ADAM-15	$\alpha_V\beta_3$	haematopoietic	Nath et al., 1999
	$\alpha_5\beta_1$	haematopoietic	Nath et al., 1999
	$\alpha_9\beta_1$	oocytes	Eto et al., 2000
ADAM-17	$\alpha_5\beta_1$	epithelial	Bax et al., 2004
ADAM-23	$\alpha_V\beta_3$	neuroblastoma	Cal et al., 2000
ADAM-28	$\alpha_4\beta_1$	lymphocytes	Bridges et al., 2002
	$\alpha_9\beta_1$	T cell leukemia	Bridges et al., 2005
	$\alpha_4\beta_7$	T cell leukemia	Bridges et al., 2005
ADAM-33	$\alpha_9\beta_1$	T cell leukemia	Bridges et al., 2005

Table 5. Cell-surface MMP-associated proteins. Many of the functions and binding mechanisms of these comlexes have not been elucidated yet. ADAM, a disintegrin and metalloproteinase; α_2-M, α_2-macroglobulin; BS, bone sialoprotein; DMP-1, dentin matrix protein-1; FPR, formyl peptide receptor; Gp, membrane glycoproteins; Hsp, heat shock protein; HSPG, heparan sulfate proteoglycans; PAR1, protease-activated receptor 1; RECK reversion-inducing cysteine-rich protein with kazal motifs.

Also, a chaperone heat shock protein90 (Hsp90) was found to interact with MMP-2 on the cell surface of fibrosarcoma cells, thus promoting MMP-2 activation, which is critical for tumour invasiveness (Eustace et al., 2004). The binding mechanism of most of these interactions has not been yet elucidated.

Several cell surface hyaluronan receptor CD44 isoforms, RECK, TSP-1, LRP, and cell surface collagen IV chains also serve as MMP-9-docking molecules. The CD44/MMP-9 complex was found to be associated with invasiveness of mouse mammary carcinoma and human melanoma cells *in vivo* (Yu and Stamenkovic, 1999), suggesting that CD44 helps to localize MMP-9 activity to the cell surface. Disruption of the CD44/MMP-9 complex by a soluble CD44 molecule resulted in the loss of tumor invasiveness and survival mediated via latent transforming growth factor-beta (TGF-β) activation. The GPI-linked proteins RECK and TSP-1 were not only identified as cell surface receptors for MMP -9 but also were found to block their enzymatic activity (Oh et al., 2001; Rodriguez-Manzaneque et al., 2001). Recombinant purified RECK was also known to inhibit the activity of MT1-MMP *in vitro* (Oh et al., 2001). When the expression of RECK was restored in cells which lack the endogenous protein, the invasive, metastatic, and angiogenic phenotypes of these tumor cells were strongly suppressed (Takahashi et al., 1998; Oh et al., 2001; Liu et al., 2003). TSP-2 is capable of regulating the proteolytic activity of MMP-2, forming a tight complex that induces scavanger receptor-mediated endocytosis and clearance (Yang et al., 2001). Interaction of MMPs with the cell surface may not only be needed for proenzyme activation and targeting at specific sites for degradation of cell-surface substrates, but could also promote intracellular degradation via receptor-mediated endocytosis (RME). For example, the LDL receptor-related protein, LRP is capable of inducing RME of MMP-2, -9, and -13, three MMPs known to have a key role in promoting tumour invasion and metastasis (Emonard et al., 2004; reviewed in Emonard et al., 2005). MT1-MMP can regulate such interactions as it has been described to proteolytically process CD44 (Kajita et al., 2001) and more recently, LRP (Rozanov et al., 2004), a possible mechanism utilized by tumor cells to maintain an invasive phenotype. Regulation of the cell surface activity of proteolytic enzymes that are involved in cancer progression, including MMP-2, -9, -13, tPA, and uPA by endocytosis has led to suppression of tumour cell invasion (Czekay et al., 2001). Both MMP-2 and -9 were found bound to the surface of various cell lines via a surface-associated α2(IV) chain of collagen type IV (Olson et al., 1998; Toth et al., 1999). ProMMP-9 bound to α2(IV) collagen chain with a high affinity and this interaction is thought to be mediated by the collagen-binding domain as TIMP-1, known to recognize the hemopexin domain, had no effect on this interaction. However, the hemopexin domain of proMMP-9 was recently shown to be important for binding gelatin (Roeb et al., 2002). MMP-8, MMP-19, and other proteases also localize to the cell surface by a mechanism that has not yet been determined.

A Disintegrin and a Metalloproteinase (ADAMs) and ADAM with a thrombospondin motif (ADAMTS) are large family of proteins capable of interacting with integrins and involved in processes such as angiogenesis, fertilization, myogenesis, neurogenesis, and inflammation. Unlike the transmembrane proteins ADAMs, ADAMTS proteins are soluble ECM proteases consisting of a prodomain, metalloprotease and disintegrin domains, but devoid of ADAMs' cystein-rich, EGF-like, transmembrane and cytoplasmic domains (reviewed in Primakoff and Myles, 2000). ADAM2 or fertilin β was one of the first disintegrin identified and found to interact with $\alpha_6\beta_1$ integrin (Chen et al., 1999). Based on knockout mice studies,

ADAM2 was found to have an important role in mediating sperm-egg adhesion (Cho et al., 1998). To date, several other ADAM-integrin interactions have been identified: ADAM9 with $\alpha_v\beta_5$ and $\alpha_6\beta_1$, ADAM12 and ADAM15 with $\alpha_9\beta_1$, ADAM15 and ADAM23 with $\alpha_v\beta_3$, and ADAM15 with $\alpha_5\beta_1$ (reviewed in Evans, 2001) (Table 5).

ROLE OF INTEGRINS AND GELATINASES IN CANCER PROGRESSION

Early events in tumor progression are characterized by increases in cell proliferation, insensitivity to growth-inhibitory signals, reduced ability for differentiation, as well as the ability to escape from apoptosis and immune surveillance (Hanahan and Weinberg, 2000). Proteinases that degrade components of the ECM and are capable of processing nonmatrix substrates (e.g. growth factors and its receptors, chemokines, adhesion molecules, and apoptotic mediators) have long been considered to be important at all stages of tumorigenesis (Coussens and Werb, 2001). The combined participation of integrins and MMPs is required for invasion of tumor cells into surrounding connective tissues, intravasation and extravasation from blood vessels, and metastasis to distant organs (reviewed in McCawley and Matrisian, 2000). Indeed, studies on TIMPs have shown that overexpression or administration of these inhibitors as recombinant proteins inhibited experimental invasion and metastasis (Alvarez et al., 1990; Khokha, 1994; Codony-Servat et al., 1999; Turpeenniemi-Hujanen, 2005). In most cases, the stage of tumor progression correlates with the expression levels of gelatinases as the invasive and metastatic potential of tumor cells is strongly affected by changes in gelatinase expression in animal models. MMP levels can appear elevated even in the early stages of tumor progression (Stetler-Stevenson, 1996). Expression of MMP-2 and MMP-9 was found to be strongly upregulated in cancers of lung, colon, breast, skin, and prostate which correlated with increased tumor invasiveness and metastasis (reviewed in Egeblad and Werb, 2002). Inhibition of MMP-9 expression in a model of experimental metastasis reduced the number of colonies formed in the lung of mice (Hua and Muschel, 1996). Further evidence supporting this hypothesis came from studies on MMP-2 and -9 null mice. These mice developed fewer tumors than the wild-type (reviewed in Sternlinct and Werb, 2001).

INTEGRINS AND GELATINASES IN TUMOR ANGIOGENESIS AND GROWTH

Angiogenesis, the formation of new blood vessels from pre-existing ones is essential for tumor growth (Hanahan and Folkman, 1996). Growth and metastasis of many cancers require the incorporation of vascular and lymphatic endothelial cells from pre-existing neighbouring vessels (Folkman, 1995; He et al., 2004). Among MMPs, gelatinases are important positive regulators of angiogenesis as both their natural and synthetic MMP inhibitors have the ability to decrease vessel sprouting into tumors in several experimental models *in vitro* and *in vivo* (Schnaper et al., 1993; Itoh et al., 1998; Vu et al., 1998; Gatto et al., 1999; Oh et al., 2001; Li et al., 2001). The role of gelatinases in tumor angiogenesis is also supported by the tumor-specific efficacy of MMP inhibitors in several transgenic models. For example, MMP-9 has been shown to be important for angiogenesis in the K14-HPV16 skin (Coussens et al., 2000) and RIP-TAg pancreatic islet

(Bergers et al., 2000) tumor progression models. The authors demonstrated that MMP-9 regulates tumor angiogenesis by releasing the pro-angiogenic factor VEGF from ECM and makes its available for binding to its receptors. Mobilization of VEGF was inhibited by TSP-1, an MMP-9 inhibitor and potential endogenous suppressor of capillary morphogenesis *in vivo*, which resulted in decrease of tumor growth by an antiangiogenic mechanism (Rodriguez-Manzaneque et al., 2001). Interestingly, MMP-2 did not seem to be required for angiogenesis in the RIP-TAg model, but strongly reduced tumor development. However, downregulation of MMP-2 expression decreased angiogenesis in a chicken chorioallantoic membrane model (Fang et al., 2000). Studies on MMP-2- (Itoh et al, 1998) and MMP-9- (Vu et al., 1998) null mice show impaired tumor angiogenesis and growth compared with the wild-type. Cleavage of type IV collagen triggered by MMP-2 exposes a cryptic, $\alpha_v\beta_3$ integrin binding site within the collagen. Indeed, an antibody recognizing the site strongly inhibited angiogenesis *in vitro* and tumor growth *in vivo* (Xu et al., 2001). In addition, epitope exposure correlated with proMMP-2 activation and increase of $\alpha_v\beta_3$ binding, suggesting that interaction between the cryptic site of collagen IV and $\alpha_v\beta_3$ could promote MT1-MMP/MMP-2 activation and eventually, increased tumor growth and invasion. In addition, MT1-MMP (Zhou et al., 2000; Galvez et al., 2001) and cathepsins (Joyce et al., 2004) have been able to trigger the angiogenic switch, suggesting the existance of important positive regulators other than MMP-9.

Gelatinase-induced cleavage of matrix components releases fragments with anti-angiogenic activity. For example, MMP-2 and MMP-9 cleave plasminogen generating angiostatin, whereas MMP-3 and MMP-9 generate a fragment from the basement membrane collagen type XVII, called endostatin. These fragments inhibited endothelial cell proliferation and tumor growth (O'Reilly et al., 1994; 1997). Another fragment, tumstatin is generated by MMP-9 through cleavage of the α_3 chain of collagen IV (ColIVα3). This fragment has been shown to be a ligand for $\alpha_v\beta_3$, an integrin highly expressed in blood vessels that are present in large tumors. It has also been detected in the blood circulation, and recently, found to be acting as angiogenesis inhibitor and tumor suppressor (Kalluri, 2003; Hamano et al., 2003). The authors detected lower levels of this fragment in the circulation of MMP-9-deficient mice which led to increased growth of large tumors. Shedding of SPARC (Secreted Protein Acidic and Rich in Cysteine), a protein that modulates cellular interaction with the ECM, by MMP-3 produced peptides capable of regulating endothelial cell proliferation and migration (Sage et al., 2003). We can conclude that MMP-9 has both pro- and anti-angiogenic properties, as it is capable of not only promoting tumor growth, but also restraining the growth of tumors by generating endogenous inhibitors of angiogenesis.

The α_v integrins play an important role in primary tumor growth in human melanoma (Felding-Habermann et al., 1992). Endothelial cell integrins, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ (Kim et al., 2000) have also been essential for regulating angiogenesis. Although $\alpha_v\beta_3$ and $\alpha_v\beta_5$ inhibitors blocked tumor angiogenesis in various models (Stupack and Cheresh, 2002), lack of these integrins did not block developmental angiogenesis in β_3 integrin knockout mice (Reynolds et al., 2002), suggesting that endothelial cell integrins, as well as MMPs, can regulate tumor angiogenesis both positively and negatively.

INTEGRINS AND GELATINASES IN INVASION AND METASTASIS

The initial step of tumor cell invasion is characterized by the breakdown of the basement membrane, a process known to be dependent on type IV collagen-degrading enzymes, mainly MMP-2 and MMP-9. Liotta obtained results where type IV gelatinase activity correlated with cancer metastasis (Liotta et al., 1980). Endothelial cell proliferation and migration into the tumor tissue is mediated by angiogenic (ex. MMP-9, VEGF, and bFGF) and lymphangiogenic factors that are released by tumor cells. During metastasis, tumor cells must dissociate from the primary tumor, degrade the ECM and enter the blood or lymph circulation by a process called intravasation. Tumor cells that have escaped from the immune system will have to break through blood vessel and lymph vessel walls, this time by a process called extravasation, into the surrounding tissue where they must be able to grow in a new environment (secondary tumor) (Mignatti and Rifkin, 1993). Using DNA microarrays, primary tumor-gene expression profiles could be arranged in classes of "good" and "poor" prognosis (Sorlie et al., 2001). Kang and colleagues performed DNA-microarray analysis on human breast carcinoma cell lines that have metastasized to bone. These studies revealed some of the genes (ex. MMP-1, MMP-2, CXCR4, IL-11, and CTGF) responsible for the increased metastatic potential of breast cancer cells (Kang et al., 2003; Minn et al., 2005). Videomicroscopy studies revealed that MMPs play a significant role in tumor metastasis, as TIMP-1 and MMP inhibitor batimastat (BB-94) blocked the formation of tumors in secondary sites (reviewed in Chambers et al., 2002). The role of MMPs in tumor invasion and metastasis has also been studied using antisense technology. For example, antisense expression of MMP-9 resulted in decreased invasiveness of human glioblastoma cells (Kondraganti et al., 2000). Also, injections of a replication-deficient adenovirus capable of expressing antisense uPAR and MMP-9 transcripts led to the inhibition of invasion, tumor growth, and metastasis of non-small cell lung cancer cells (NSCLC) (Rao et al., 2005). RNA interference-directed knockdown of uPA and its receptor uPAR, and MMP-9 inhibited invasion and growth of prostate and glioma cells *in vivo*, respectively (Murali-krishna et al., 2005; Lakka et al., 2005). MMP-2 induced expression also correlate with high metastatic potential of prostate carcinomas (Stearns and Stearns, 1996). Furthermore, downregulation of the endogenous MT1-MMP gene expression using RNA silencing technology also inhibited the invasion of highly invasive fibrosarcoma HT1080 cells (Ueda et al., 2003). Gelatinases and MT-MMPs revealed a new mechanism to control metastasis by cleavage of the metastasis suppressor gene, KiSS-1 (Takino et al., 2003). Finally, recent studies supporting the *in vitro* data from double MMP-2:MMP-9-deficient mice demonstrated that these enzymes cooperate in promoting the invasive phenotype of malignant keratinocytes in an experimental model *in vivo* (Masson et al., 2005). Also, the number of metastatic colonies in MMP-9 deficient mice was significantly decreased in an experimental in vivo model where mice were injected intravenously with melanoma or lung carcinoma cells (Itoh et al., 1999).

Changes in integrin expression and localization can also influence invasion and metastasis of tumor cells (Maschler et al., 2005). Integrins were shown to be involved in the migration and liver metastasis of large cell lymphoma cells and angiogenesis, as $\alpha_v\beta_3$ antagonists induced apoptosis and blocked cancer cell

invasion (Brooks et al., 1994). $\alpha_4\beta_1$ integrin has a dual role in cancer progression as it inhibited the initial invasive growth while promoting metastatic spread of melanoma cells. A different study showed that increased expression of this integrin could inhibit the invasive stage of metastasis formation (Qian et al., 1994). Overexpression of $\alpha_2\beta_1$ integrin had no effect on primary tumor growth but stimulated both experimental and spontaneous metastasis (Chan et al., 1991). Blocking integrins with synthetic peptides containing an RGD sequence, antibodies, or disintegrins (integrin-binding proteins isolated from snake venom) has been demonstrated to intefere with tumor cell invasion and metastasis *in vitro* and *in vivo* (Gehlsen et al., 1988; Curley et al., 1999). Importantly, cooperation between $\alpha_v\beta_3$ and MMP-9 increased migration of metastatic breast cancer cells (Rolli et al., 2003). Increased expression of MMP-9 and $\alpha_V\beta_6$ integrin promoted epithelial cell migration during tumor formation via a novel TNF-α-dependent mechanism (Scott et al., 2004). Also, several reports show that uPA binding to its receptor, uPAR is a requirement for tumor cell invasion and metastasis as this process is efficiently inhibited either by an amino-terminal fragment of urokinase or a mutant plasminogen activator inhibitor-2 (PAI-2) (Wang et al., 2005). Chemokines can also be involved in organ-specific metastatic growth of tumor cells. This hypothesis is supported by a recent study which highlights the importance of chemokine receptors in breast cancer metastasis *in vitro* and *in vivo*. For example, treatment of animals with a neutralizing anti-human CXCR4 monoclonal antibody inhibited cancer cell metastasis to lung tissue (Muller et al., 2001).

INTEGRINS AND GELATINASES IN CANCER-ASSOCIATED INFLAMMATION

Chronic inflammation is also associated with a variety of cancers, including breast, liver, prostate, and skin (reviewed in Coussens and Werb, 2001). In human cancer, tumor cells are not the only source of MMPs. MMPs, mainly gelatinases are predominantly produced by stromal cells, ranging from immune (lymphocytes and dendritic cells), inflammatory (granulocytes and monocytes), and vascular cells (vascular- and lymph-endothelial cells and pericytes). MMPs have been involved in the escape of cancer cells from immune surveillance. The escape mechanism occurs through MMP-9-induced cleavage of the interleukin-2 receptor-α (IL-2Rα) (Sheu et al., 2001), TGF-β activation (Yu and Stamenkovic, 2000), and ICAM-1 and ICAM-2 shedding (Mustjoki et al., 2001; Fiore et al., 2002; Sultan et al., 2004), thus suppressing T cell proliferation and immune response against tumors.

Chemokines play an essential role in regulating directional migration of leukocytes. Proteolytic cleavage of chemokines by MMPs can lead to enhanced or reduced leukocyte recruitment into tumors. For example, MMP-9 activates the neutrophil chemoattractant IL-8 by cleavage, while it inactivates the CXCL7 precursor, the platelet factor-4 (PF-4), the growth-related oncogene-α (GRO-α), and stromal cell-derived factor 1 (SDF-1) (reviewed in Egeblad and Werb, 2002). A cleaved form of MCP-3 produced by MMP-2 can bind to CC-chemokine receptors, and unlike intact MCP-3, it abrogates chemotaxis and suppresses inflammation (McQuibban et al., 2000). ET-1 processing by MMP-9 generates endothelin-1 (ET-1) that induces secretion of MMP-9 from neutrophils (Fernandez-Patron, 2001), suggesting that MMPs are both

effectors of leukocyte migration and regulators of the inflammatory response. The importance of chemokine receptors in metastasis was demonstrated by inhibition of SDF-1-binding to its receptor. Dissociation of SDF-1/CXCR-4 complex by blocking antibodies strongly reduced breast cancer metastasis to lungs and lymph nodes *in vivo* (Müller et al., 2001). MMP-9 and VEGF are produced by mammary tumor-infiltrating immune cells (Owen et al., 2003). Expression of MMP-9 by tumor-infiltrating macrophages promotes angiogenesis as well as growth and invasion of xenografted ovarian cancer cells *in vivo* (Huang et al., 2002). Several studies show that cancer cells can promote the secretion of MMPs by stromal cells in a paracrine manner via secretion of growth factors, interleukins, and EMMPRIN (reviewed in Sternlinct and Werb, 2001). Various data have shown that recruitment of hematopoietic precursor cells is required for tumor angiogenesis (Coussens et al., 2000). Indeed, transplantation of MMP-9-expressing bone marrow cells into MMP-9$^{-/-}$ mice increased tumor growth, probably by delivery of MMP-9-producing monocytes into the tumor vasculature.

ROLE OF INTEGRINS AND GELATINASES IN ACUTE LEUKEMIAS

Leukemia can be described as the uncontrolled proliferation of hematopoietic cells that lack the ability to differentiate into mature blood cells. The precise role of gelatinase-expression in acute leukemias is not clear. So far, it is known that invasiveness of many hematological malignancies, including myelo-monocytic leukemias involves overexpression of proteolytic enzymes, such as the MMP-2 and MMP-9 (Janowska-Wieczorek et al., 1999; Kuittinen et al., 1999; Klein et al., 2001). MMP-9 is induced and secreted in conditioned media of leukemic cell lines in response to extracellular stimuli, after pretreatment of cells with chemokines, and after cell adhesion to the ECM (Xie et al., 1998; Wize et al., 1998; Klein et al., 2001; Takafuji et al., 2003). Higher gelatinase-expression levels were detected in the bone marrow plasma of patients with leukemia compared with normal controls. After chemotherapy, the levels of TIMP-1 and TIMP-2 were significantly increased, whereas MMP-9 levels were lower in ALL and AML patients. Accordingly, AML patients who achieved a complete remission showed significantly lower MMP-9 levels, suggesting that MMP-9 could be a surrogate marker of leukemic status in these patients. Also, the low MMP-9 expression levels in patients with leukemia correlated with increased survival (Lin et al., 2002).

Several reports have demonstrated the involvement of both MMP-2/-9 gelatinases and β_2 integrins in the growth and progression of myeloid and lymphoid neoplasms (Kossakowska et al., 1993; Noguchi et al., 2001; Hayashibara et al., 2002). Selective MMP-9 expression is induced as a result of $\alpha_M\beta_2$ integrin ligation in PMNs (Wize et al., 1998) and $\alpha_L\beta_2$ integrin ligation in T lymphoma cells (Aoudjit et al., 1998). Also, studies from α_M and α_L integrin knockout mice confirm the importance of β_2 integrins in mediating leukocyte adhesion and migration (Bouvard et al., 2001). In accordance, high infiltration of leukemic blasts in patients with AML strongly correlated with increased expression of both $\alpha_L\beta_2$ and $\alpha_M\beta_2$ integrins (Noguchi et al., 2001). AML cell adhesion to bone marrow fibroblast monolayers seems to require both β_1

and β_2 integrins, as antibodies against them inhibited the binding (Liesveld et al., 1993; Bendall et al., 1993; Stucki et al., 2001). Interaction between leukemic cells and bone marrow stroma cells has been shown to increase leukemic cell survival and chemotherapy-induced leukemia cell resistance (Garrido et al., 2001).

Increased vessel density was detected in the bone marrow of acute and chronic leukemia patients compared with normal bone marrow, and known to be mediated by angiogenic factors such as VEGF and basic fibroblast growth factor (bFGF) (Werb, 1997; Aguayo et al., 2000). Both increased plasma MMP-9 and VEGF correlated with high leukemia cell infiltration, suggesting that MMP-9 and VEGF act co-operatively in the process of leukemia cell invasion (Hayashibara et al., 2002). Another study showed that increased vessel density was mediated by MMP-2 and MMP-9 overexpression in primary AML blasts by promoting endothelial cell migration (de Bont et al., 2001). After achieving complete remission, the vessel number in AML patients was restored to normal levels. Furthermore, a gene therapy approach using a retroviral vector encoding for gelatinase inhibitors, endostatin and angiostatin strongly inhibited bone marrow angiogenesis and leukemia tumor growth *in vivo* (Scappaticci et al., 2001). These data suggest that gelatinases could be involved in leukemia progression. As a result, inhibitors of MMPs may be useful in treating hematological malignancies.

THERAPEUTIC INTERVENTION WITH MMP AND INTEGRIN INHIBITORS

Due to the fact that integrins and MMPs are involved in tumor cell invasion and metastasis, a lot of effort has been put over the past 20 years in designing integrin and MMP inhibitors (MMPIs). Although endogenous inhibitors, such as TIMPs inhibited tumor growth in transgenic mouse models, their use in cancer was limited due to poor pharmacokinetics, difficulties in protein administration, and broad spectrum of inhibition. To date, several synthetic MMPIs have been developed, tested widely in clinical trials, and classified into the following pharmacological groups: collagen peptidomimetics, non-peptidomimetics, tetracycline derivatives, and biphosphonates (reviewed in Hidalgo and Eckhardt, 2001; Ala-Aho and Kähäri, 2005). The efficacy of these inhibitors in clinical trials is summarized in Table 6.

The design of collagen peptidomimetic MMP inhibitors is based on the collagen-peptide backbone with zinc-binding hydroxamate moiety that coordinates the Zn^{2+} ion, thus inhibiting the MMP catalytic activity. Batimastat was the first peptidomimetic inhibitor used in clinical trials for the treatment of malignant tumors but is no longer used in clinical trials as it could not be administered orally. However, treatment with marimastat, an oral MMPI variant of batimastat, significantly increased survival of patients with gastric carcinoma (Bramhall et al., 2002). Treatment with marimastat was well tolerated by the patients, except some small side effects translated with musculoskeletal pain, probably because of the need of MMPs in normal remodelling of the connective tissue of tendons and joints. In patients with advanced pancreatic cancer (a phase II study), marimastat showed comparable therapeutic effects as conventional therapy with gemcitabine that was used (Bramhall et al., 2002). The survival of patients suffering from glioblastoma

multiforme was also improved by using marimastat in combination with temozolomide, a cytotoxic drug (Groves et al., 2002). Several non-peptidomimetic MMP inhibitors, including BMS-275291, AG3340, and MMI270 have also been tested in clinical trials (Table 6).

Tetracyclines and biphosphonates have also been shown to block MMP activity (Boissier et al., 2000; Cianfrocca et al., 2002). For example, a broad spectrum MMP inhibitor, metastat (or Col-3) showed increased tumor cell toxicity, reduced tumor-induced angiogenesis, as well as antimetastatic activity (Seftor et al., 1998; Lokeshwar et al., 2002), and is currently being tested in patients with Kaposi Sarcoma and brain cancer in a phase II clinical trial. Periostat, a tetracycline used for the treatment of periodontal diseases, is the only MMPI in the market.

Inhibitors	Structure	Specificity	Status/Indication
MMP			
Batimastat (BB-94)	Peptidomimetic	MMP-1, -2, -3, -7, -9	Development halted
Marimastat (BB-2516)	Peptidomimetic	MMP-1, -2, -7, -9	Phase III/Gastric cancer Phase II/Pancreatic cancer
BAY12-9566	Nonpeptidomimetic	MMP-2, -3, -9	Development halted
AG3340	Nonpeptidomimetic	MMP-2, -3	Phase II/III/No benefit
BMS-275291	Nonpeptidomimetic	Broad spectrum	Phase I/II/NSCL
MMI270	Nonpeptidomimetic	Broad spectrum	Phase I/Advanced cancer
Metastat (Col-3)	Tetracycline derivative	MMP-2, -9	Phase II/Kaposi sarcoma
Periostat	Tetracycline derivative	Broad spectrum	Phase M/Periodontal disease
Neovastat (AE-941)	Shark cartilage extract	Broad spectrum	Phase II/Multiple myeloma Phase III/NSCL
-	Green tea extract	MMP-2, -9	Phase III/Cancer
Integrin			
Efalizumab/ Hu1124	Humanised MAb	CD11α subunit	Phase III/Psoriasis
Anti-CD18	Humanised MAb	CD18	PhaseII/ Myocardial infarction
Anti-LFA1	Murine	CD18	Phase III/Allograft rejection
Hu23F2G	Humanised MAb	CD11/CD18 integrin	Phase II/Multiple sclerosis Phase II/Myocardial infarction Phase III/Stroke
LDP-01	Humanised MAb	CD18 integrin subunit	Phase II/Allograft rejection /Stroke
LDP-02	Humanised MAb	$\alpha_4\beta_7$ integrin	Phase II/Ulcerative colitis
Volociximab & Erlotinnib	MAb	$\alpha_5\beta_1$ integrin	Phase II/metastatic NSCL
ATN-161	PHSRN motif from FN	$\alpha_5\beta_1$ integrin	Phase I/NSCL
M200	MAb	$\alpha_5\beta_1$ integrin	Phase II/Kidney cancer
Vitaxin / LM609	Humanised MAb	$\alpha_V\beta_3$ integrin	Phase II/Sarcoma
Antegren	Humanised MAb	$\alpha_4\beta_1$ integrin	Phase III/Multiple sclerosis PhaseII/Colitis,Crohn's disease
Tysabri/Natalizumab	MAb	$\alpha_4\beta_1$ integrin	Phase M/Multiple sclerosis
Abciximab	Chimeric Ab	$\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_M\beta_2$	FDA approved
Eptifibatide	Cyclic heptapeptide	$\alpha_{IIb}\beta_3$ integrin	FDA approved
Tirofiban	Peptidomimetic	$\alpha_{IIb}\beta_3$ integrin	" " /Myocardial infarction
Cilengitide	Cyclic RGD peptide	$\alpha_V\beta_3$/$\alpha_V\beta_5$ integrins	Phase II/GBM
Altocor/ Lovastatin	Chemical	$\alpha_L\beta_2$ integrin	FDA approval/atherosclerosis

Table 6. MMP and integrin antagonists in clinical trials. NSCL, non-small-cell lung cancer; FN, fibronectin; Phase M, on the market; ; GBM, glioblastoma multiforme; Mab, monoclonal antibody; FDA, food and drug administration.

Interestingly, compounds (TSRI265) capable of inhibiting interactions between MMPs and integrins showed promising results in animal experiments (Silletti et al., 2001). Also, a cyclic peptide, CTTHWGFTLC discovered by phage display technology as a selective gelatinase inhibitor could block cell migration and tumor growth in a gelatinase-dependent manner (Koivunen et al., 1999).

The failure of MMPIs in several cancer clinical trials is not surprising (reviewed in Coussens et al., 2002). Most MMPIs were used to treat patients in late-stage tumors, whereas most results obtained from animal experiments show the need for targeting MMPs in early stages of cancer progression. Also, these inhibitors target all MMPs, many of which are needed for the processing of anti-angiogenic factors, including angiostatin and endostatin. For that, increasing the selectivity of these compounds (for example, for gelatinases involved in metastasis) could solve the problem of side effects reported so far. MMPIs are known to target also ADAMTS, enzymes capable of reducing tumor growth by blocking tumor angiogenesis (Vazquez et al., 1999). It should be taken in consideration that other proteases are upregulated during tumor progression that could compensate the loss of MMPs. These proteases should be identified and targeted along with MMPs.

The involvement of integrins in tumor cell invasion and metastasis became clear after using α_V (Eliceiri and Cheresh, 1999) or β_1 (Senger et al., 2002) subunit-blocking antibodies or small synthetic antagonists generated from the ligand's-recognition sequence. Humanized mAbs, Vitaxin and Efalizumab against $\alpha_V\beta_3$ (Posey et al., 2001) and α_L subunit of $\alpha_L\beta_2$ (Lebwohl et al., 2003) respectively, and the synthetic, cyclic Arg-Gly-Asp (RGD) peptide motif (Dechantsreiter et al., 1999) present in many integrin ligands were the three among many other integrin-binding agents that have entered cancer clinical trials (see Table 6). Efalizumab and a recombinant mAb against $\alpha_4\beta_1$, natalizumab have shown a great promise in the treatment of psoriasis (Lebwohl et al., 2003), as well as in multiple sclerosis and Crohn's disease, respectively (Miller et al., 2003; Ghosh et al., 2003). However, β_3 and β_5-integrin knockout mice showed increased expression of VEGFR-2 receptor, leading to enhanced tumor angiogenesis (Reynolds et al., 2002). Taken together, MMP and integrin knockout models and inhibitors can increase our understanding of the multiple functions of these molecules in several diseases, including cancer. Such studies may be used to develop therapeutic agents which can interfere with the integrin and MMP function on invasive tumor cells and blood vessels.

AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. To study whether β_2 integrins could act as receptors for MMP-2 and MMP-9 on the surface of leukocytes and leukemic cell lines, and by which binding mechanism.

2. To identify peptides by phage display technology capable of inhibiting the interaction between leukocyte integrins and MMPs.

3. To study the role of the integrin/proMMP complexes in leukocyte migration *in vitro* and *in vivo*.

4. To identify small-molecule compounds that bind to integrins, which could be more suitable for drug development.

5. To determine the role of the $\alpha_M\beta_2$ integrin/MMP-9 complex as a functional target for inhibition of leukemia cell dissemination and growth *in vivo*.

MATERIALS AND METHODS

Detailed descriptions of the materials and methods are found in the original publications.

Materials and Methods	Original Publications
Antibodies, synthetic peptides, cell lines	**I-IV**
Peptide biosynthesis and chemical synthesis	**I**
Cell culture and neutrophil isolation	**I-IV**
Purification of leukocyte integrins	**I, II**
Expression and purification of integrin I domains and MMP-9 recombinant proteins	**I-IV**
Enzyme-linked immunosorbent assay (ELISA)	**I, II**
SDS-PAGE and immunoblotting	**I-IV**
Coprecipitation experiments	**I**
Immunoprecipitation	**I**
Cell adhesion and Transwell migration	**I-IV**
Transendothelial migration/Chemotaxis assay	**II**
Immunohistochemical staining of tissues	**IV**
Gelatin zymography	**I, II**
Confocal microscopy	**I, II, IV**
Phage display pannings	**I**
Phage binding assays	**I**
Subcellular fractionation	**II**
Cell surface labeling using ^{125}Iodine and periodate-tritiated sodium borohydride	**II, IV**
Cell surface biotinylation	**IV**
MMP-9 cell surface release	**I**
Binding of MMPs to purified integrins	**I**
Pepspot	**I**
Metabolic radiolabeling	**II**
Antibody production and purification	**IV**
Cell viability and proliferation assay	**III, IV**
Peptide affinity chromatography	**IV**
Cell binding assay using ^{125}I-MMP-9 domains	**IV**
RNA interference and RNAi-transfections	**IV**
Thioglycolate-induced peritonitis *in vivo*	**II**
Leukemia cell dissemination *in vivo*	**IV**
Human leukemia xenograft models *in vivo*	**IV**
Pericellular proteolysis	**IV**
Small molecule compound library screening	**III**
Statistical analysis	**II, IV**

RESULTS

Identification of DDGW, an $\alpha_M\beta_2$ integrin-binding peptide that inhibits leukocyte migration in vitro (I).

Using phage peptide display libraries, we selected peptides that interact with the α_M I domain. The consensus D/E-/D/E-G/L-W determined by this approach was clearly different from other β_2 integrin-binding peptides reported so far. We chose the peptide CILWMDDGWC (DDGW) for further experiments as this peptide-bearing phage showed strong binding to the $\alpha_{L/M}$ I domains and this was readily inhibited by low concentrations of the DDGW peptide. Data from protein databases revealed that this motif is present and is highly conserved in the catalytic domain of MMPs, including gelatinases MMP-2/-9 (Figure 1A). To find a dominant integrin-binding site in the catalytic domain of proMMP-9 was unexpected, as previous reports suggested another MMP domain to be required for integrin binding. For example, the hemopexin domains of MMP-2 and MMP-1 mediated binding to $\alpha_V\beta_3$ (Brooks et al., 1996; Brooks et al., 1998) and $\alpha_2\beta_1$ integrins (Dumin et al., 2001; Stricker et al., 2001).

No MMP has been ever reported to bind to the leukocyte β_2 integrins. We therefore set out to study whether MMP-9 in particular could be a ligand of the β_2 integrins as MMP-9 gelatinase is the major leukocyte MMP and is induced during β_2 integrin activation. Binding assays with the α_M I domain revealed a single active peptide that located to the MMP-9 catalytic domain. The sequence of the I domain-binding peptide was QGDAHFDDDELWSLGKGVVV and it contained a similar binding motif to the one identified by phage display. The active MMP-9 peptide contained four consecutive amino acids with negative charges, DDDE. Alanine scanning mutagenesis on both DDDE- and DDGW-containing peptides significantly abrogated I domain binding Figure 1E). Also, peptides derived from other known integrin ligands, including myeloperoxidase, catalase, thrombospondin-1 and complement protein iC3b strongly bound the I domain in this assay and the double alanine mutations caused a loss of binding (Table I).

Progelatinases bound in a concentration-dependent manner to coated $\alpha_M\beta_2$ integrin. The binding of gelatinases was observed with both $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins and their corresponding I domains. ProMMP-9 bound like a true integrin ligand, as the cation chelator EDTA nearly completely prevented the binding. The DDGW peptide was an efficient inhibitor and it inhibited proMMP-9 binding to the α_M I domain with an IC_{50} of 20 µM. Curiously, MMP-2 and MMP-9 lost the integrin binding ability after activation by trypsin or APMA (Figure 2). Furthermore, proMMP-9 and $\alpha_M\beta_2$ were found to co-localize on the cell surface of activated leukemia cells. Cell surface labeling and coimmunoprecipitation studies further demonstrated the occurrence of the complex in leukemic cell lines (Figure 7). We suggest that the MMP-9 co-localizing with $\alpha_M\beta_2$ is the proMMP-9, as the activated MMP-9 did not bind to $\alpha_M\beta_2$.

We then studied the effect of the DDGW peptide on adhesion and migration of human myelomonocytic THP-1 cells. Phorbol-ester activated cells efficiently bound to the DDGW peptide, whereas

there was no binding in the absence of cell activation. The acute myeloid leukemic cell line OCI/AML-3 also avidly adhered to DDGW, whereas human fibrosarcoma HT1080 cells which lack β_2 integrins did not. DDGW did not block cell adhesion to fibrinogen and ICAM-1. Both the DDGW and CTT peptides inhibited the migration of THP-1 cells on the LLG-C4-GST substratum. Unlike DDGW, CTT was also capable of inhibiting the migration of β_2 integrin lacking cells, HT1080 (Figure 9). These results suggest an important function for the integrin-progelatinase complex in leukocyte migration.

Blocking the progelatinase/β_2 integrin complex inhibits migration of PMNs to an inflammatory site *in vivo* (II)

Previously, endogenous neutrophil elastase, proteinase 3, and cathepsin G have all been reported to bind to $\alpha_M\beta_2$ in PMNs (Cai and Wright, 1996). Immunofluorescence and surface labeling with THP-1 leukemic cells from earlier experiments showed an intense intracellular colocalization of $\alpha_M\beta_2$ integrin and proMMP-9 (I; Figure 7B). Staining of resting and activated PMNs with integrin and MMP antibodies showed even more intense intracellular colocalization. After PMA-treatment to cause exocytosis of intracellular granules, the intracellular staining decreased and $\alpha_M\beta_2$ integrin and MMP-9 colocalized to the cell surface. Although proMMP-9 is known to localize to the same intracellular granules as the $\alpha_M\beta_2$ integrin, association of proMMP-9 with $\alpha_M\beta_2$ intracellularly has not been shown before.

Immonoprecipitation studies with integrin and MMP antibodies were performed on purified azurophilic-, specific-, gelatinase-, secretory vesicle- and plasma membrane-fractions from PMNs. In non-activated PMNs, the proMMP-9/$\alpha_M\beta_2$ complex was immunoprecipitated from the gelatinase granules, whereas after PMA-stimulation, it was translocated to the PMN cell surface. The biosynthesis of the endogenous complex was also investigated in the THP-1 leukemic cell line, which is amenable for such studies. The complex was detected at 2 h and 4 h time points by immunoprecipitation from [^{35}S]-methionine pulsed cells. These results indicate that the proMMP-9 association is an early event for the integrins and that the immunoprecipitated material does not represent endocytosed or recycling integrins. This is a more plausible mechanism for the MMP/integrin complex formation than binding of a secreted MMP to the integrin on the cell surface. First of all, the integrin could transport the endogenously-bound proMMP-9 to an appropriate site without competition by extracellular MMP inhibitors and integrin ligands. Secondly, as the I domain of $\alpha_M\beta_2$ does not bind active MMP-9 (I, Figure 2A), the integrin could regulate the timing of proMMP-9 activation and release of the active enzyme.

Experiments with recombinant MMP-9 domains gave further support for our finding that a site interacting with the integrin is present on the MMP-9 catalytic domain (II, Figure 4B) and we developed an active I domain binding peptide that was only six residues in length. This peptide, HFDDDE, corresponds to a linear sequence from the MMP-9 catalytic domain and efficiently competed with proMMP-9 binding to $\alpha_M\beta_2$ (IC_{50} of 20 µM) or its purified I domain. ICAM-1 and fibrinogen did not compete with either proMMP, implying different binding sites for the matrix proteins and proMMPs in the I domain.

Then, we investigated the effect of the HFDDDE peptide on the adhesion and migration of PMNs. After integrin activation, PMNs exhibited an ability to adhere on proMMP-9. Cell adherence was inhibited by HFDDDE (50µM), DDGW (50µM), the soluble α_M I domain and the MEM170 antibody, indicating β_2 integrin-directed binding. Similar results were obtained using the $\alpha_M\beta_2$ L-cell transfectants. The *in vitro* migration of PMNs was studied either with transwell or transendothelial layer assays. Chemotaxis with C5a or TNF-α increased PMN transmigration by 5-10 fold and inhibition was obtained either by DDGW, HFDDDE, CTT or α_M and MMP-9 antibodies (II, Figure 6).

To study neutrophil migration *in vivo*, we used a mouse model of thioglycolate-induced peritonitis. Approximately 20-fold more PMNs were present intraperitoneally after thioglycolate-stimulus in comparison to the PBS control. The effects of DDGW and HFDDDE peptides were concentration-dependent and up to 80 % inhibition was obtained by doses of 0.1mg/kg and 4 mg/kg per mouse, respectively. Inflammatory PMNs from the pritoneal cavity were stained positively for the proMMP-9/$\alpha_M\beta_2$ complex, whereas cells collected after PBS injection lacked the complex; they expressed the integrin but had no cell-surface MMP-9. DDGW and HFDDDE prevented the increase in gelatinase levels present in the peritoneal cavities in accordance with the inhibition of cell migration (II, Figure 7). Taken together, these results suggest that the proMMP-9/$\alpha_M\beta_2$ complex may play a role in PMN motility *in vitro* and *in vivo*.

Identification of an $\alpha_M\beta_2$ integrin–small binding molecule which inhibits integrin-dependent leukemia cell migration (III)

We developed an assay for the identification of small-molecules that interact and compete with a DDGW peptide-bearing phage for binding to the α_M I domain of $\alpha_M\beta_2$ integrin. By this technique, we were about to select compounds that bind to the target protein with a high affinity, are more stable against proteolysis and less susceptible to rapid clearance *in vivo*. The most active compound, IMB-10 bound six times stronger to both α_L and α_M I domains than the peptide and inhibited DDGW phage binding. Surprisingly, unlike DDGW, IMB-10 failed to disrupt the interaction between the I domains and proMMP-9. However, IMB-10 was capable of stabilizing the active conformation of the I domain as it increased the binding to integrin ligands (Figure 2). In addition, $\alpha_M\beta_2$ integrin-expressing cells adhered strongly to coated integrin ligands in the presence of IMB-10 and failed to detach in the presence of cation-chelating agents, such as EDTA (Figure 4). Furthermore, single amino acid point mutations in the integrin α_M I domain indicated that Lys245 would be an important residue in mediating binding to progelatinases. Also, mutations in the hydrophobic pocket accommodating the C-terminal α-helix, which is known to regulate integrin's high affinity state conformation, inhibited the I domain's ability to be induced by IMB-10.

Finally, IMB-10 showed no signs of toxicity at concentrations of 25 µM and efficiently inhibited integrin-dependent migration when added to THP-1 and OCI-AML-3 cells *in vitro* (Figure 5). This compound had no effect in mobilization of cells that lack leukocyte β_2 integrins. Similarly, the function of IMB-10 was also tested in a thioglycolate-induced peritonitis model *in vivo* (Figure 6). The number of

leukocytes that were recruited to the peritoneum was reduced by 70 % at three hours post-induction of the inflammation.

Inhibition of human leukemia tumor xenografts by blocking the interaction between integrins and progelatinases (IV)

We recently demonstrated that proMMP-9 bound via its catalytic domain to α_M and α_L I domains of β_2 integrins on the surface of leukemic cell lines (I, Figure 2B). This binding was inhibited by the MMP-9-related peptide, HFDDDE derived from the MMP catalytic domain sequence, and by the β_2 integrin ligand, DDGW obtained by phage display (II, Figure 4C). Next, we determined the affinity of proMMP-9 to the cell surface using recombinant catalytic and C-terminal hemopexin domains. ^{125}I-ΔproMMP-9 bound to OCI-AML-3 cells with high affinity [dissociation constant (K_d) = 93 nM ± 18 nM; n=3]. Inhibition experiments with the α_M and α_L antibodies, and integrin-binding peptides indicated that $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins serve as receptors for ΔproMMP-9. We also used short interfering RNAs (siRNA) to suppress the endogenous α_M integrin gene on OCI-AML-3 and THP-1 cells. ^{125}I-ΔproMMP-9 binding to the RNAi-treated cells was significantly reduced as compared to untreated cells. One of the α_M siRNAs suppressed integrin expression as shown by protein levels at 72 h post-transfection and by FACS. α_M siRNAs did not affect proMMP-9 protein expression levels. RNAi-treated cells showed weakened binding to ICAM-1- or proMMP-9-coated surfaces indicating the loss of integrin function. Taken together, these data indicate that β_2 integrins, possibly both $\alpha_M\beta_2$ and $\alpha_L\beta_2$, anchor the proMMP-9 catalytic domain to the cell surface (IV, Figure 3).

As the peptides efficiently inhibited leukemia cell migration in several *in vitro* assays (I, Figure 9B; II, Figure 6B), we set out to study the role of MMP-integrin complexes in leukemia cell growth and dissemination in mouse models. Exposure of human OCI-AML-3 and THP-1 leukemic cells *in vitro* to either HFDDDE or DDGW peptides at concentrations as high as 500 µM showed no signs of cytotoxicity. However, when administered to mice, the peptides significantly suppressed the growth of OCI-AML-3 and THP-1 xenografts, the linear HFDDDE hexapeptide being consistently more active than the cyclic 18-mer DDGW. Nude mice were injected subcutaneously with human OCI-AML-3 cells and divided into 5 groups (n = 8 mice/group). Peptides were administered i.v. at day 4 postinoculation and at concentrations of 10 mg/kg 5 times a week. The HFDDDE- and DDGW-treated mice had significantly smaller tumors at day 20 compared to sequence related but inactive control peptide groups. The tumor suppressive effect of HFDDDE lasted longer than that of DDGW and at day 160, when the experiment was ended, 3 out of 8 tumor-bearing mice in the HFDDDE group showed tumor regression. Treatment with HFDDDE and DDGW peptides led to a statistically significant increase in survival that was extended by 140 days and by 50 days, respectively. Similar results were obtained with the peptides, this time by treating THP-1 tumor-bearing mice (IV, Figure 1).

Several reports demonstrate that tumor-infiltrating leukocytes and increased tumor angiogenesis contribute to the survival, growth, and progression of various malignant tumors (Folkman et al., 1995; Lin

and Pollard, 2004). We therefore checked whether the integrin-binding peptides had any effect on host's immune system and tumor vascularization. Histological examination of tumor sections with an antibody against mouse $\alpha_M\beta_2$ integrin showed diminished host infiltrating leukocytes and blockage of blood vessel formation in HFDDDE- and DDGW-treated tumors compare to saline-treated. As the subcutaneously growing leukemias may not properly mimick human leukemias which disseminate via the blood, we next examined leukemia dissemination in mice at approximately 40 days i.v. postinoculation of OCI-AML-3 cells. Mice subjected to HFDDDE-peptide treatment showed decreased tumor burden and lower number of visible tumor nodules in the liver, lungs, and gastrointestinal system (IV, Figure 1). In a different mouse model, the HFDDDE peptide and an antibody raised against it inhibited the infiltration of ^{125}I -labeled OCI-AML-3 cells to the liver, bone marrow, and spleen. The HFDDDE peptide also inhibited proMMP-9 binding to the surface of AML cells, blocked cell-mediated gelatin degradation, and "stabilized" several cell surface proteins, including β_2 integrins. In addition, the MMP-9 levels were significantly increased in the serum of mice which have been treated with the integrin-binding peptides, HFDDDE and DDGW (IV, Figure 2). These results indicate that disruption of the integrin/MMP complexes presented here provides an experimental framework to yield AML-targeted therapies..

DISCUSSION

Extensive effort has been made in developing small molecules, peptides and peptidomimetics capable of inhibiting interactions which occur on the cell surface. Several linear and cyclic peptides derived from sequences of β_2 integrins, ICAMs, and ECM proteins have been shown to have inhibitory effects *in vitro* and *in vivo*. Indeed, ICAM-1-derived peptides can control immune responses in autoimmune diseases and allograft rejection by simply blocking ICAM-1-binding to $\alpha_L\beta_2$ integrin. Peptides derived from the sequence of β_1- and β_2-integrins have been also shown to be potent anti-inflammatory agents by blocking integrin-mediated adhesion of leukocytes (Yusuf-Makagiansar et al., 2002). Furthermore, inhibition of an integrin-MMP cell-surface complex, $\alpha_V\beta_3$/MMP-2 dramatically suppressed angiogenesis *in vivo*, suggesting that this interaction is essential for endothelial cell proliferation and migration (Silletti et al., 2001). Such reagents were reported not only to interfere with ligand binding, but also could stabilize integrin conformations. Conformational changes after ligand or peptide binding is known to induce exposure of neoepitopes referred to as ligand-induced binding site epitopes (LIBS) (Takagi et al., 1997; Lu et al., 2001b). Finally, integrin-directed small molecules have entered phase I and II clinical cancer trials as they showed strong inhibition of tumor angiogenesis (Kerbel et al., 2000). Also, peptides containing the RGD sequence have been demonstrated to inhibit experimental tumor metastasis in animal models (Curley et al., 1999).

In our study, a cyclic peptide DDGW discovered by phage display, both inhibited proMMP-9/$\alpha_M\beta_2$ complex formation and leukocyte migration *in vitro* and *in vivo*. However, this motif did not block leukocyte adhesion to ICAM-1 and fibrinogen, suggesting the integrin-bound MMP is essential for degradation of integrin-directed bonds to matrix proteins. We also discovered a small molecule recognizing the $\alpha_M\beta_2$ integrin which had no effect on the integrin/MMP complex formation but instead it enhanced the binding of the integrin I domain to its ligands by stabilizing the active conformation of the I domain.

Studies on the role of MMP-9 in leukocyte migration have been controversial. For example, some reports have supported MMP-9 function in leukocyte migration (Keck et al., 2002; Lee et al., 2003), whereas others have not (Betsuyaku et al., 1999; Allport et al., 1999). These findings are not surprising as MMPs are known to have overlapping functions and other MMPs within the family could compensate for the loss of MMP-9.

The molecular basis of the integrin-MMP interaction was first studied *in vitro* using purified integrins or integrin domains and purified MMPs, both in ELISA and immunoprecipitation experiments. Cell surface labeling and coimmunoprecipitation studies further demonstrated the occurrence of the complex in leukemic cell lines. Experiments with recombinant MMP-9 domains gave further support for our finding that the site interacting with the integrin is present on the MMP-9 catalytic domain (Figure 6; left panel). The putative binding site was revealed by using synthetic peptides as 20-mers, spanning the entire MMP-9 sequence.

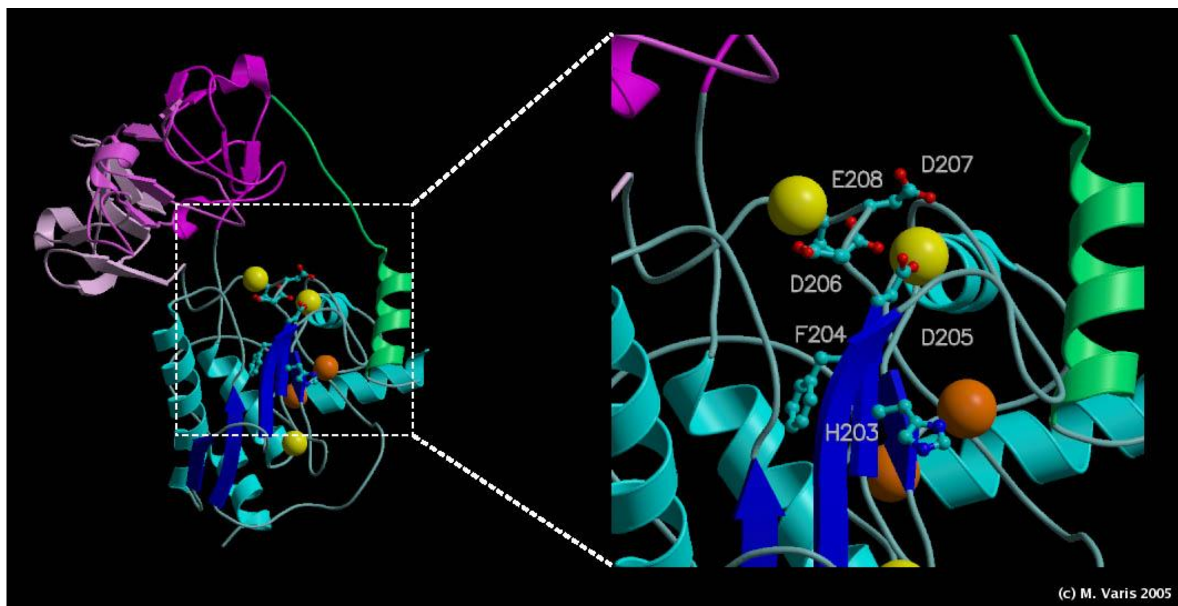


Figure 6. Structure of the C-terminally truncated proMMP-9. The pro-domain and catalytic domain, as well as the three fibronectin type II (FnII) repeats are shown in green, cyan/blue, and light pink, light magenta, and magenta, respectively. The HFDDDE peptide motif, located in the catalytic domain is shown in a higher magnification (right panel) and amino acids are drawn as ball-and-stick models. The blue β strands and cyan α helices belong to the catalytic domain of pro-MMP-9. Calcium atoms are depicted in yellow and zinc atoms in orange. The proMMP-9 pictures were generated using Molscript and Raster3D programs. Gimp-2.2 (http://www.gimp.org) has been used to modify the pictures from the PDB entry 1L6J (Elkins et al., 2002). The figure is printed with the permission of Minna Varis.

The binding motif in these peptides was homologous to our phage display integrin-binding peptide and to a sequence element, HFDDDE found in the catalytic domain of MMP-2 and -9 (Figure 6; right panel). This motif could also be involved in chelating the two Ca^{2+} ions which are coordinated by the negatively charged amino acids, D^{205} and E^{208}. Although the approach to mimick features of complex protein structure with small peptides may appear somewhat simplified, our study came up with peptides capable of blocking the interaction between MMP-9 and $\alpha_M\beta_2$ integrin. However, we can exclude the possibility that these peptides also affect other β_2 integrin ligands than proMMP-9.

A very interesting finding was the observation that the proenzyme, but not the activated MMP bound to the integrin. Although mechanistically unclear (the site identified to mediate the binding in MMP-9 is not within the prodomain), this feature could be important for the control of MMPs activity *in vivo*. Also, the location of the binding site being close to the MMP catalytic center further suggests a mechanism for evading inhibition by TIMPs or α_2-macroglobulin. In the absence of inhibitors, the cell surface-localized proMMP-9 would be readily susceptible for activation and substrate hydrolysis, which can occur in the presence of the intact propeptide (Bannikov et al., 2002). In addition, our results suggest that proMMP-9 binds preferentially to the extended conformation of the integrin as the integrin-binding compound, known to stabilize the active conformation of the α_M I domain strongly increased its binding to proMMP-9. It remains to be seen whether other integrins conformations, such as the bent (inactive) and intermediate forms (Xiong et al., 2001; Takagi et al., 2002) can support proMMP-9 binding.

Moreover, we present evidence that proMMP-9/$\alpha_M\beta_2$ complex is stored within the intracellular granules in resting PMNs and translocated to the cell surface upon cell stimulation (Figure 7). This is a more plausible mechanism for the MMP/integrin complex formation than binding of a secreted MMP to an unoccupied integrin on the cell surface. Also, leukocyte integrins could play a role in targeting of proMMPs to a site where proteolytic activity is needed. However, it remains to be determined by which mechanism (pro)MMP-9 is located at the surface of cells lacking β_2 integrins.

Activation of cells with cytokines, chemokines, and chemical agents is known to induce the release of transmembrane proteins, including TGF-α, TNF-α, and L-selectin (Arribas et al., 1996; Fiore et al., 2002), a process that is known to be essential for regulating the cellular functions of these receptors. It has been shown that proteolytic processing of fibrinogen regulates $\alpha_M\beta_2$ integrin-mediated leukocyte adhesion and detachment on this substrate (Lishko et al., 2002). The $\alpha_M\beta_2$ integrin ligands ICAM-1 and fibrinogen, which are important for leukocyte migration act also as substrates for MMP-9 (Lelongt et al., 2001; Fiore et al., 2002; Sultan et al., 2004). Accordingly, collagenolysis by MMP-1 is known to be required for efficient migration of keratinocytes on type I collagen matrix (Pilcher et al., 1997). Finally, MT1-MMP-mediated shedding of laminin-5 leads to stimulated epithelial cell migration (Koshikawa et al., 2000).

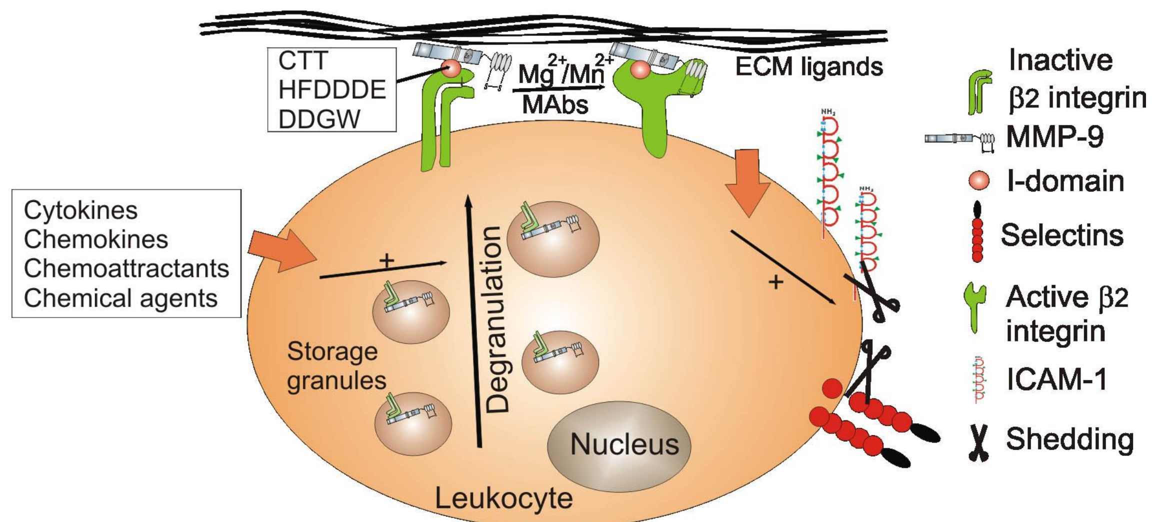


Figure 7. Schematic summary of the integrin/MMP complex in PMNs. The $\alpha_M\beta_2$/MMP-9 complex is formed in PMN intracellular granules and can be rapidly mobilized to the cell surface after exposure to degranulation stimuli, such as TNF-β, LPS, and fMLP. PMN degranulation can also be achieved when cells are in contact with ECM proteins. Upon PMN activation, cell-surface receptors are routinely shed from PMNs. Loss of these receptors is mainly due to PMN-derived MMP activity, a process that facilitates PMN rolling and migration via degradation of the vascular basement membrane during PMN extravasation.

The physiological role of MMP-2 and -9 is not fully understood, but to our current knowledge they are involved in the processing of the extracellular matrix during growth and tissue differentiation, probably as critical factors for cell motility. Proteases and integrins for such a function have been expected to be

colocalized at the surface of migrating cells. Most MMPs, however, are secreted enzymes and the search for cell surface receptors for MMPs has been going on for years. At the moment there are some hundred publications describing receptors, such as integrins for various MMPs, among them MMP-2 and -9. Likewise, gelatinase activity has been found in the membrane of leukocytes, but the identification of the leukocyte integrins as gelatinase receptors is new to our knowledge and likely to extend our understanding of further mechanisms involved in leukocyte migration. Studies from knockout models for integrins, including leukocyte β_2 integrins confirm their involvement in various steps of cancer development. Eventually, tumor growth and metastasis could be blocked by interfering with integrin function on tumor cells and blood vessels. This study provides evidence that a small, negatively charged peptide, derived from the catalytic domain of MMP-9, efficiently blocks $\alpha_M\beta_2$/MMP-9 complex formation, leukemia cell-mediated pericellular proteolysis, leukemia cell extravasation and growth *in vivo*. Several MMP inhibitors have been developed and tested in clinical trials but the results have been disappointing. Based on our findings, new and more effective cancer therapeutics could be achieved by blocking, not only MMPs alone but their association with integrins or other cell surface receptors. Selective antagonists of the MMP-9/$\alpha_M\beta_2$ integrin interaction may not only be therapeutic in leukemias but also in other types of malignancies where tumor-infiltrating leukocytes enhance tumor growth.

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