Institut für Molekularbiologie
(Tumorforschung)

Molecular Mechanisms of Hematogenous Tumor Metastasis

INAUGURAL DISSERTATION

zur Erlangung des Doktorgrades
der Naturwissenschaften
(Dr. rer. nat.)
des Fachbereichs
Biologie und Geographie
an der Universität Duisburg-Essen

vorgelegt von

Constantin Adams, Wittmund

13. Juni 2007

Die der vorliegenden Arbeit zugrundeliegenden Experimente wurden am Institut für Molekularbiologie (Tumorforschung) der Universität-Gesamthochschule Essen durchgeführt.

1. Gutachter: Prof. Dr. Erich Gulbins

2. Gutachter: Prof. Dr. Michael Ehrmann

Vorsitzende des Prüfungsausschusses: Prof. Dr. Ann Ehrenhofer-Murray

Tag der mündlichen Prüfung: 17. Oktober 2007

In Memoriam

Joyce Green

(1931-2004)

List of Abbreviations

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ADP	adenosine diphosphate
AGR	G-protein-coupled receptors
APAF-1	apoptotic protease activating factor-1
ASM	acid sphingomyelinase
cAMP	cyclic adenosine monophosphate
CD62P	cluster of differentiation 62 (synonym for P-selectin)
DAG	diacylglyceride
DMSO	dimethyl sulfoxide
DNA	dioxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDG1	endothelial differentiation gene 1
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
GPIIbIIIα	glycoprotein IIbIIIα
HEV	high endothelial venules
HPLC	high pressure liquid chromatography
Ig	immunoglobulin
IL-1	Interleukin-1
JNK	c-Jun n-terminal kinase
kDa	kilodaltons
KSR	kinase suppressor of Ras
LDL	low-density lipoprotein
MAPK	mitgen-activated protein kinase

NADPH	nicotine amide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NOS	nitric oxide synthase
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PKC	protein kinase C
PLCγ	Phospholipase C γ
PP1	protein phosphatase 1
PP2A	protein phosphatase 2 A
P-sel	P-selectin
PSGL-1	P-selectin glycoprotein ligand 1
ROS	reactive oxygen species
SAPK	stress-activated phospho-kinase
SD	standard deviation
Smac	second mitochondria-derived activator of caspases
TLC	thin layer chromatography
TNF-α	Tumor necrosis factor α
UV	ultraviolet
vWF	von Willebrandt Factor

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NOS	nitric oxide synthase
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PKC	protein kinase C
PLCγ	Phospholipase C γ
PP1	protein phosphatase 1
PP2A	protein phosphatase 2 A
P-sel	P-selectin
PSGL-1	P-selectin glycoprotein ligand 1
ROS	reactive oxygen species
SAPK	stress-activated phospho-kinase
SD	standard deviation
Smac	second mitochondria-derived activator of caspases
TLC	thin layer chromatography
TNF-α	Tumor necrosis factor α
UV	ultraviolet
vWF	von Willebrandt Factor

Summary

The present manuscript demonstrates that B16F10 melanoma cells activate the enzyme acid sphingomyelinase in thrombocytes via the surface molecule P-selectin, by which ceramide is released. Metastasis of tumor cells in the lung is decreased by up to 95% by genetic deficiency of P-selectin molecule or deficiency of acid sphingomyelinase. After activation of wild type thrombocytes by B16F10 melanoma cells there is a rapid increase in acid sphingomyelinase activity and ceramide production as compared to acid sphingomyelinase-deficient thrombocytes or P-selectin-deficient thrombocytes. A lack of interaction of B16F10 melanoma cells and thrombocytes was excluded by activation of PLCγ, JNK and MAP kinase, indicating that these signaling events are stimulated in both, wild-type and P-selectin-deficient platelets, proving that B16F10 melanoma cells interact with and activate P-selectin-deficient thrombocytes.

The molecular mechanisms of tumor metastasis are currently fairly incomplete, though metastasis plays a crucial clinical role in cancer patients. Acid sphingomyelinase is iidentified as a novel target molecule for the inhibition of tumor metastasis. In order to pharmacologically inhibit the thrombocytic P-selectin system, an intravenous injection of fucoidan showed a decrease of tumor metastasis of B16F10 melanoma cells by approximately 75%. This indicates that tumor metastasis can be blocked pharmacologically, which is of great clinical interest.

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Essen, den 11. Juni 2007

List of Publications

Rompaey LV, Potter M, Adams C, Grosveld G. **Tel induces a G1 arrest and suppresses Ras-induced transformation.** Oncogene. 2000 Nov 2;19(46):5244-50.

Buijs A, van Rompaey L, Molijn AC, Davis JN, Vertegaal AC, Potter MD, Adams C, van Baal S, Zwarthoff EC, Roussel MF, Grosveld GC. **The MN1-TEL fusion protein, encoded by the translocation (12;22)(p13;q11) in myeloid leukemia, is a transcription factor with transforming activity.** Mol Cell Biol. 2000 Dec;20(24):9281-93

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Bock J, Szabo I, Gamper N, Adams C, Gulbins E. **Ceramide inhibits the potassium channel Kv1.3 by the formation of membrane platforms.** Biochem Biophys Res Commun. 2003 Jun 13;305(4):890-7.

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Szabo I, Adams C, Gulbins E. **Ion channels and membrane rafts in apoptosis.** Pflugers Arch. 2004 Jun;448(3):304-12. Epub 2004 Apr 8. Review

Szabo I, Bock J, Jekle A, Soddemann M, Adams C, Lang F, Zoratti M, Gulbins E. **A novel potassium channel in lymphocyte mitochondria.** J Biol Chem. 2005 Apr 1;280(13):12790-8. Epub 2005 Jan 4.

Kirschnek S, Adams C, Gulbins E. **Annexin II is a novel receptor for Pseudomonas aeruginosa. Biochem Biophys Res Commun.** 2005 Feb 18;327(3):900-6.

Constantin Adams, M.S. (USA)

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Fachbereiche 6 und 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Molecular Mechanisms of Hematogenous Tumor Metastasis“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Constantin Adams befürworte.

Essen, den 11. Juni 2007

Prof. Dr. Erich Gulbins

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Fachbereiche 6 und 9 zu Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 11. Juni 2007

Constantin Adams

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 und 9 zu Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den 11. Juni 2007

Constantin Adams

1. Introduction

1.1 Tumor Cell- Adhesion Molecule Interaction

1.1.1 Overview of tumor metastasis

Most human cancers are of epithelial origin, and most deaths that result from these carcinomas are caused by tumor spread to distant organs, a process known as tumor metastasis (Kim *et al.*, 1998). Tumor metastasis is a multistep cascade during which tumor cells have to cross the local extracellular matrix, penetrate the vascular endothelium, circulate in the blood (or lymph), and extravasate through vessel walls into the tissue for the formation of secondary tumors. During this process, tumor cells associate with cells like leukocytes, platelets, or endothelial cells, acquisition of properties that allow this association are of advantage (Friederichs *et al.*, 2000). It may be of particular importance for tumor cells to associate with platelets. Karpatkin *et al.* (1988) and Mehta *et al.* (1987) have shown that tumor cells can circulate in the vasculature as stabilized platelet- enriched thrombi that can physically protect tumor cells from destruction, while the ability to bind to endothelial cells may also be an advantage of the tumor cells. Tumor cells, once in the vasculature, have to bind to the endothelial lining of the vessel wall, which is an essential step for tumor extravasation and eventual tissue penetration. A general overview is depicted in Fig. 1.

Fig. 1

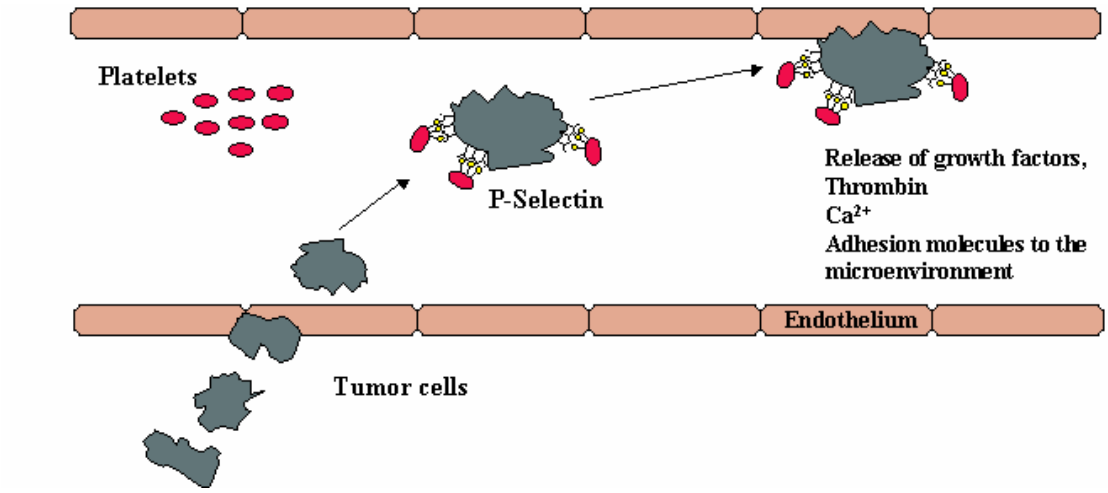


Fig. 1: General mechanism of tumor metastasis.

Primary tumor cells migrate from the site of origin, enter the blood circulation and interact with platelets to form neoplastic emboli that can arrest in the microvasculature and adhere to the endothelium. This adhesion is in part mediated through the action of selectins. Interaction of tumor cells with selectins prevents tumor cell lysis by the immune system.

1.1.2 Role of P-selectin in tumor metastasis

Tumor cell adhesion to platelets, leukocytes or endothelial cells is in part mediated by selectins, a family of cell surface carbohydrate- binding proteins (McEver *et al.*, 1997; Krause *et al.*, 1999; Kannagi *et al.*, 2004). Three types of selectins have been discovered thus far. L-selectins (CD62L) are generally expressed on almost all leukocytes. E-selectins (CD62E) are inducible on vascular endothelium upon stimulation with cytokines due to transcriptional activation. P-selectins (CD62P) were originally found on activated platelets, but their expression is also induced on activated vascular endothelium. P-selectins are stored as preformed transmembrane proteins in cytoplasmic granules (Weibel-Palade bodies of endothelial cells and α-granules of platelets) which are translocated onto the cell surface upon stimulation by cytokines such as Il-1 and TNF-α (Frenette *et al.*, 2000). L-selectin mediates

adhesion to result in formation of leukocyte-tumor cell emboli as well as local secretion of cytokines and growth factors which are thought to aid in secondary tumor growth (Qian *et al.*, 2001; Borsig *et al.*, 2002; Rosen *et al.*, 2004), while E- and P-selectin on the endothelium facilitate tumor cells and emboli to anchor in the microvasculature (McEver *et al.*, 1997; Krause *et al.*, 1999; Kannagi *et al.*, 1997; Hartwell *et al.*, 1999). P-selectin ligands have been detected previously on several human carcinomas and cell lines (Mannori *et al.*, 1995; Aruffo *et al.*, 1992; Stone *et al.*, 1993).

Under conditions not involving tumor cells, leukocyte recruitment is integral to host defense, during which endothelial selectins mediate tethering (initial attachment), rolling of leukocytes for halting circulation of the immune cells to extravasate to inflamed tissue (McEver *et al.*, 1995) by binding counter-receptor carbohydrate ligands expressed on the surface of leukocytes, and weak attachment of the leukocytes onto the vessel wall. Integrins react with the Ig superfamily of cell adhesion molecules for firm adhesion and signal transduction, triggering diapedesis and the transendothelial migration of leukocytes (Chen *et al.*, 2006). This combined action of selectins provides a mechanism that enables tumor cells to survive and to populate distant organs (Brown *et al.*, 2006).

Functionally, leukocyte rolling serves two main purposes, the successful recruitment of neutrophils, monocytes, eosinophils, some effector T-cells and dendritic cells to the site of acute and chronic inflammation, and the successful exit of T- and B-lymphocytes from high endothelial venules (HEVs) into the parenchyma of secondary lymphoid organs (Sperandio, 2006). Leukocyte rolling is an important step in the recruitment of leukocytes into tissue and has been considered to be a rather nonspecific process that allows leukocytes to obtain contact with the vascular wall (Sperandio, 2006). During rolling, leukocytes have the opportunity to screen the endothelial surface for specific trigger signals, leading to a decision for extravasation into tissue.

Drastic changes during malignant transformation occur with cell surface carbohydrate determinants, and it is well known that expression of sialyl Lewis-x and sialyl Lewis-a determinants is significantly enhanced in cancer cells. These

determinants are ligands for the selectins present on endothelial cells and they also mediate hematogenous metastasis of tumors.

As a ligand for P-selectin, a mucin-like carbohydrate-presenting molecule P-selectin glycoprotein ligand 1 (PSGL-1) has been reported (Frenette *et al.*, 2000), which has been subject of intensive investigation in the past few years (Vestweber and Blanks, 1999) and was first identified on myeloid cells. PSGL-1 is a homodimer or two disulfide-linked subunits of approximately 120 kDa (Moore, 1998; Yang *et al.*, 1999) that carries O-linked sialyl-Le-x glycans (Moore *et al.*, 1992; Moore *et al.*, 1994). PSGL-1 is expressed on human myeloid HL-60 cells, neutrophils, lymphocytes (Moore *et al.*, 1995), $CD34^+$ early progenitor cells (Zanettio *et al.*, 1995) and other hematopoietic cells, though not always in an active form (Vachino *et al.*, 1995). Cloning of PSGL-1 showed a mucin- like protein with several potential sites of O-linked carbohydrates and tyrosine posphorylation (Sako *et al.*, 1993). PSGL-1 requires both the specific glycoconjugates sialic acid and fucose, and the sulfated tyrosine rsidues for the calcium-dependent recognition by the P-selectin lectin domain (Sako *et al.*, 1995; Pouyani and Seed, 1995). Because PSGL-1 mediates leukocyte rolling at physiological shear (Norman *et al.*, 1995), it is a good candidate to also mediate platelet rolling. Frenette *et al.* (2000) showed that PSGL-1 mRNA and glycoprotein are expressed in mouse and human platelets, and that function-blocking antibody against murine PSGL-1 can inhibit selectin-mediated platelet-endothelial interactions *in vivo*. Further natural ligands for P-selecin include heparin and heparan sulfate proteoglycans (Geng, 2003). Aigner *et al.* (1998) have identified and characterized another ligand for P-selectin: human CD24 on a breast and a small lung carcinoma cell line, KS and SW-2, respectively, can bind to P-selectin under static conditions (Aigner *et al.*, 1997). CD24 is a mucin-like glycosylphosphatidylinositol-linked cell surface glycoprotein, consisting of a small protein core of only 27 extensively glycosylated amino acids (Aigner *et al.*, 1995), bound to the membrane via a phosphatidyl inositol anchor (Pirruccello and LeBien, 1986; Fischer *et al.*, 1990; Kay *et al.*, 1991). Potential sites for O-linked glycosylation are present in nearly half of the CD24 amino acids, composed by serine and threonine. CD24 is highly expressed at the early stages of B-cell development, on neutrophils and many human carcinomas, but is absent on T-cells, monocytes or normal adult tissue (Akashi *et al.*, 1994; Jackson *et al.*, 1992; Pirruccello and LeBien,

1986). Aigner *et al.* (1998) showed that CD24 can function as a physiological counter receptor for P-selectin and mediate rolling of the breast carcinoma cell line KS.

1.1.3 Fucoidan as a ligand for P-selectin

In arterial thrombogenesis, plaque rupture followed by the formation of a thrombus is a fundamental pathophysiology of acute coronary syndromes (Fuster *et al.*, 1988), in which platelets are a major component of these thrombi. Current treatment includes anti-platelet drugs like aspirin, and inhibition of specific platelet integrin receptor glycoprotein IIb/IIIα has been shown to inhibit platelet aggregation and thrombus formation by the prevention of platelet-platelet interaction (Shattil *et al.*, 1985. Yokoyama *et al.* (2005) have shown that inhibition of P-selectin function by monoclonal antibody to P-selectin reduces platelet-mediated thrombus formation (Ueyama *et al.*, 1997; Ikeda *et al.*, 1999), and in a canine model the incorporation of platelets with upregulated P-selectin to leukocytes in large arterial thrombi of acute coronary syndromes was shown (Ikeda *et al.*, 1999). Therefore, platelet P-selectin plays an important role in arterial thrombogenesis by forming large stable platelet-leukocyte aggregates. Thorlacius *et al.* (2000) showed that the polysaccharide fucoidan has anticoagulative properties in terms of thrombin clotting time prolongation, and it has been suggested that this anticoagulative effect is mainly due to enhanced thrombin inhibition by different mechanisms (Colliec *et al.*, 1991; Church *et al.*, 1989; Nishino *et al.*, 1991). Other references propose the anticoagulative effect being due to fucoidan primarily stimulating the release of tissue factor pathway inhibitor from endothelial cells (Giraux *et al.*, 1998). Fucoidan is known to bind and interfere with the activity of both P- and L-selectin (Bevilacqua and Nelson, 1993; Nelson *et al.*, 1993), thereby causing the antithrombotic effect of fucoidan *in vivo*. Thorlacius *et al.* (2000) show that fucoidan effectively prevents microvascular thrombus formation induced by endothelial damage in arterioles and venules *in vivo*; however, this protective effect of fucoidan cannot be attributed to the inhibition of either P-selectin or L-selectin function but instead may be related to the anticoagulative capacity of fucoidan. Fucoidan drastically delayed the progression of thrombus growth and the time required for complete vessel occlusion.

1.1.4 P-Selectin in inflammation and thrombosis

Inflammation and thrombosis play major roles in atherosclerosis pathogenesis. Activated endothelial cells release Weibel-Palade bodies, granules that contain procoagulant and proinflammatory substances including multimeric von Willebrand factor (vWF), P-selectin, and CD63 (Wagner *et al.*, 1982; Bonfanti *et al.*, 1989; Vischer and Wagner, 1993; Datta and Ewenstein, 2001; Wagner, 1993). Interleukin-8 (IL-8), thromboplastinogen, calcitonin gene–related peptide, and endothelin have been reported in Weibel-Palade bodies as well (Utgaard *et al.*, 1998; Ueda *et al.*, 1992; Ozaka *et al.*, 1997; Schaumburg-Lever *et al.*, 1994). Exocytosis of Weibel-Palade bodies is triggered by a variety of agonists, including thrombin, histamine, complement, leukotrienes, superoxide anions, epinephrine, adenosine, and vasopressin (Birch *et al.*, 1992; Hamilton and Sims, 1987; Hattori *et al.*, 1989; Vischer *et al.*, 1995; Vischer and Wollheim, 1997). Weibel–Palade body exocytosis is mediated by calcium, cAMP, and G-proteins (Birch *et al.*, 1992; Hamilton and Sims, 1987; Vischer and Wollheim, 1997; Klarenbach *et al.*, 2003). After Weibel–Palade body exocytosis, P-selectin is translocated to the endothelial surface, where it facilitates leukocyte rolling, and vWF is released into the vessel lumen, where it mediates platelet adhesion and aggregation (Ramos *et al.*, 1999; Andre *et al.*, 2000).

1.2 Sphingomyelin, Ceramide and Sphingosine-1-Phosphate

1.2.1 Overview of ceramide function

Sphingolipids found in plasma membranes of all eukaryotic cells play an important signaling role in vascular inflammation and thrombosis (Hannun and Obeid, 2002; Auge *et al*., 2000; Levade *et al*., 2001). The sphingolipid ceramide mediates a variety of stress responses (Hannun, 1996). Ceramide is produced *de novo* from serine condensation with palmitoyl-coenzyme A in the endoplasmic reticulum or from hydrolysis of sphingomyelin by sphingomyelinase in the plasma membrane, lysosomes, or endoplasmic reticulum (Huwiler, 2000). A variety of factors induce ceramide production, including tumor necrosis factor-α (TNF-α), IL-1ß, interferon-γ, CD95 ligand, B7, CD40 ligand, oxidized low-density lipoprotein (LDL), ischemia, radiation, and chemotherapeutic agents (Hannun and Obeid, 2002; Auge *et al*., 2000; Levade *et al*., 2001; Hannun, 1996). Ceramide and its metabolites mediate inflammation, thrombosis, apoptosis, cell differentiation, proliferation, and vasomotor regulation by changing the biophysical properties of the cell membrane and the distribution of receptors in the membrane as well as by acting on a variety of phosphatases, proteases, kinases, phospholipases, and transcription factors (protein phosphatase [PP] 1, PP2A, cathepsin, protein kinases, endothelial differentiation gene receptors, NF-κB, mitogen-activated protein kinase, plasminogen activator inhibitor-1 (PAI-1), nicotinamide adenine dinucleotide phosphate oxidase, and endothelial nitric oxide synthase [NOS]) (Kupatt *et al*., 1997; Qian *et al*., 2001; Hannun and Obeid, 2002; Auge *et al*., 2000; Levade *et al*., 2001; Hannun, 1996; Igarashi *et al*., 1999; Dantas *et al*., 2003; Gonzalez *et al*., 2002; Kou *et al*., 2002; Igarashi *et al*., 2001a; Igarashi *et al*., 2001b; Igarashi and Michel, 2000). Ceramide can induce vascular inflammation by activating transcriptional pathways, but ceramide can also rapidly trigger vascular inflammation by nontranscriptional pathways, although the precise mechanism by which it does so is unclear.

1.2.2 Ceramide-enriched membrane platforms

The sphingomyelin pathway is a signaling system that is conserved from yeast to humans (Peña *et al*., 1997; Hannun, 1996; Spiegel *et al*., 1996; Ballou *et al*., 1996). Membrane sphingomyelin is hydrolyzed by at least three different sphingomyelinases, which are characterized by their optimal pH: acid, neutral and alkaline. The product of the enzymatic action is ceramide, which is the amide ester of the sphingoid base D-erythrosphingosine and a fatty acid ranging from C2 to C28, but usually from C16 to C26 chain length. The best-characterized sphingomyelinase is the enzyme acid sphingomyelinase. Acid sphingomyelinase is a 75 kDa protein that is post-translationally modified by glycosylation, which is necessary for its functionality (Newrzella and Stoffel, 1996; Ferlinz, 1997). As the acid sphingomyelinase is produced, at least two forms of the enzyme are formed: a lysosomal and a secretory form. An isoform that binds to the cell surface might be a third independent isoform or either lsosomal or secretory acid sphingomyelinase. Although derived from the same gene, the lysosomal and secretory forms are differently glycosylated and processed at the NH_2-terminus. It is this glycosylation pattern that most likely determines the targeting of the two enzymatic forms of the acid sphingomyelinase to different cellular compartments, and all forms of the enzyme appear to have different functions (Gulbins, 2003). In acidic compartments like late endosomes and lysosomes the enzyme regulates sphingomyelin turnover, where the secretory form is involved in stress, inflammation and regulation of serum lipoproteins (Schissel *et al*, 1996; Schissel *et al*., 1998a; Schissel *et al*., 1998b; Tabas, 1999). The surface form of the enzyme is important for the regulation of receptor and signalling protein distribution involved, for instance, in apoptosis. This form has been shown to appear on the cellular surface upon cellular stimulation by CD95 (Grassmé *et al*., 2001a; Grassmé *et al*., 2001b) or CD40 (Grassmé *et al*, 2002). CD95 is a pro-apoptotic molecule, and several pro-apoptotic molecules like TNF-R or stress stimuli like UV-light (Chatterje and Wu, 2001) or irradiation (Santana *et al*., 1996; Pena *et al*., 1996; Paris *et al*., 2001) have been shown to activate the acid sphingomyleinase rapidly (within seconds to minutes), resulting in the release of extracellularly oriented ceramide and the formation of ceramide-enriched membrane platforms (Fig. 2 A+B).

Fig. 2 A:

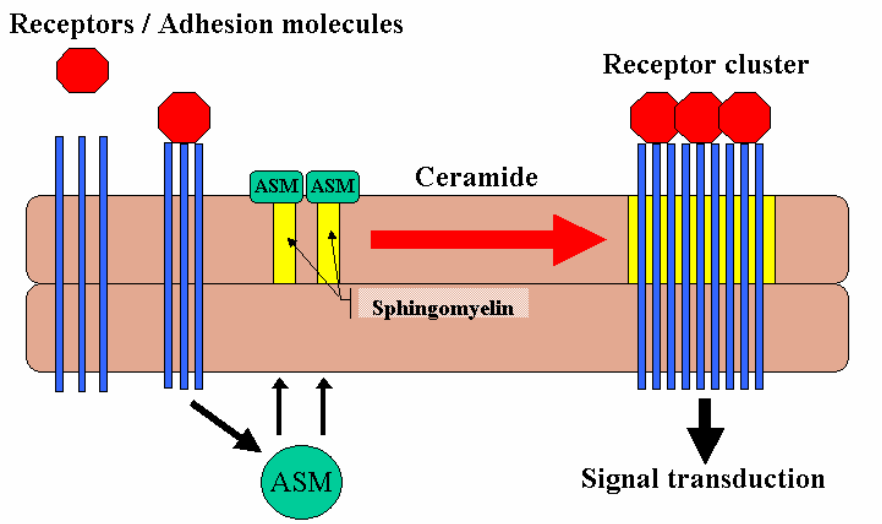

Receptors / Adhesion molecules
Receptor cluster
ASM
ASM
Ceramide
Sphingomyelin
ASM
Signal transduction

Fig. 2 B:

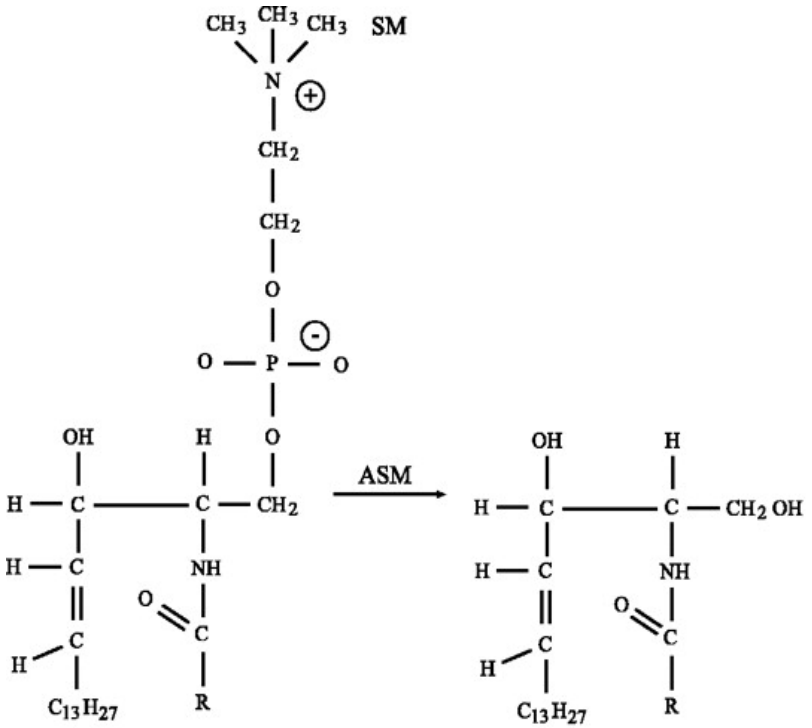

SM
ASM
Sphingomyelin
Ceramide

Fig. 2 A+B: Schematic representation of ceramide-enriched membrane platform formation through receptor clustering.

A: When stimulated, the cellular acid sphingomyelinase is activated and generates ceramide from sphingomyelin. Ceramide accumulation leads to platform formation on the cellular surface, causing aggregation of the receptors and intracellular signaling proteins within the platforms. This aggregation leads to an amplification of signal and the transmission of those signals into the cell. **B:** The chemical structure of sphingomyelin that is hydrolyzed by action of the enzyme acid sphingomyelinase, leading to the release of ceramide and phosphorylcholine (*from* Gulbins and Li, *Am J Physiol Regul Integr Comp Physiol, 2006*).

Singer and Nicholson showed (Singer and Nicholson, 1972) that membranes are not static but consist in a fluid-mosaic state, known as the fluid-mosaic model. In recent years, this model has been modified to include findings that lipids can spontaneously be organized into distinct microdomains of the cellular membrane (Anderson, 1998; Brown and London, 1998; Simons and Ikonen, 1997). The cellular membrane is mainly composed of glycerophospholipids, sphingolipids, and cholesterol. The sphingolipid headgroups cause a lateral association of lipids with one another, further enhanced by hydrophobic interactions between the saturated side chains, while cholesterol appears to fill the spaces between the large glycerosphingolipids and tightly interacts with sphingolipids, especially sphingomyelin (Gulbins and Kolesnick, 2003). This interaction results in the separation of the lipids into distinct membrane structures characterized by a liquid-ordered phase, while the glycerophospholipids in the cell membrane are located in a rather fluid liquid phase (Simons and Ikonen, 1997; Brown and London, 1998). Because sphingomyelin is almost exclusively located in the anticytosolic leaflet of biological membranes (Emmelot and Van Hoeven, 1975), rafts also appear to be present predominantly in the outer leaflet of the cell membrane and the anti-cytostolic leaflet of intracellular membranes (Gulbins and Li, 2006). Rafts- the term refers to these microdomains seemingly floating in an "ocean" of other glycerophospholipids of the cellular membrane- range in size between 30 and 300 nm, and the consumption of sphingomyelin within rafts to generate ceramide results in drastic change within these small rafts. Ceramide fuses membranes and has the ability to form large ceramide-enriched membrane platforms excluding cholesterol. Holopainen *et al*. (1998) and Nurminen *et al*. (2002) showed that ceramide generation was followed by the formation of patches

of ceramide that associated rapidly into a ceramide-enriched macrodomain, suggesting that this fusion function resulted from hydrogen bonding and van der Waal forces between ceramide molecules.

1.2.3 Ceramide generation from sphingomyelin via acid sphingomyelinase

As acid sphingomyelinase is activated by various stimuli including CD95 or CD40, infection with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, gamma irradiation or heat, the enzyme is rapidly translocated to the extracellular leaflet of the cell membrane. Gulbins and Kolesnick (2003) suggested that rapid fusion of intracellular vesicles containing acid sphingomyelinase with the cellular membrane occurs upon contact with these diverse stimuli, resulting in exposure of acid sphingomyelinase on the cellular surface. This surface acid sphingomyelinase appears to localize to rafts and releases ceramide from sphingomyelin, which in turn transforms rafts into large ceramide-enriched signaling platforms, reaching the size of several μm in diameter (Gulbins and Kolesnick, 2003). These platforms serve to trap and cluster receptor molecules like CD95 or CD40; furthermore, these ceramide-enriched membrane platforms seem to be involved in the cellular response to radiation, UV-A light, heat, some chemotherapeutics and infections with pathogenic bacteria and viruses (Gulbins and Kolesnick, 2003). Clustering of CD95 results in high local density of the receptor in a small area of the cellular surface, allowing for clustering of the receptors and eventually also of downstream effector molecules. Rapid clustering of CD95 upon stimulation has been shown in lymphocytes, phagocytic cells, epithelial cells, fibroblasts, hepatocytes and thymocytes (Fanzo *et al*., 2003).

Ceramide activates a diverse set of protein kinases and protein phosphatases (Smyth *et al*., 1997; Hannun, 1996; Ruvolo, 2001), involving the SAPK/JNK signaling pathway in apoptosis, the kinase supressor of Ras (KSR) and protein kinase C (PKC). Ceramide redirects cellular signaling pathways by promoting SAPK cascades at the expense of MAPK pathways (Jarvis *et al*., 1997). Activation of SAPK pathways induces cell cycle arrest and inhibits cell

proliferation (Bourbon *et al*., 2000), therefore ceramide generation conditions promote JNK activation (Ruvolo, 2003).

The generation of those ceramide-enriched membrane domains results in a re-organization of the cell membrane, re-ordering of receptor molecules, clustering of receptors, trapping of receptors and intracellular signaling proteins and thereby transmission of signals into the cell. Ceramide may also influence the permeability of cell membranes (Kolesnick and Kronke, 1998; Ruiz-Arguello *et al*., 1996) since ceramide can form pores that allow efflux of 20-60 kDa molecules (Siskind and Colombini, 2000; Siskind *et al*., 2002; Montes *et al*., 2002).

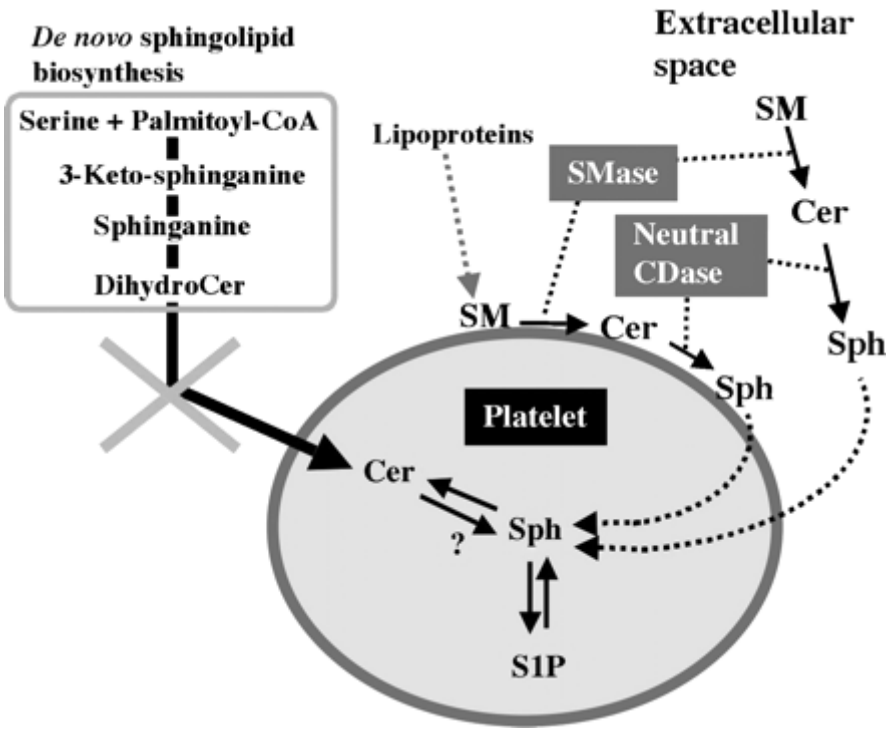
1.2.4 Sphingosine and sphingosine-1-phosphate as ceramide metabolites

The metabolites of ceramide, sphingosine and sphingosine-1-phosphate, were shown to be intracellular mediators regulating cell proliferation or apoptosis (Hannun, 1994; Hannun and Obeid, 1995; Kolesnick and Golde, 1994; Cubillier *et al*., 1996; Olivera and Spiegel, 1993; Spiegel and Milstien, 1995). Sphingosine-1-phosphate is the initial product of the catabolism of sphingosine by action of sphingosine kinase, and is then cleaved by sphingosine lyase to yield ethanolamine phosphate and a fatty aldehyde (Spiegel *et al*., 1996; Spiegel and Milstien, 1995; Van Veldhoven and Mannaerts, 1991). It has been revealed that sphingosine-1-phosphate is a ligand for G-protein-coupled receptors (AGR), endothelial differentiation gene 1 (EDG1) (Lee *et al*., 1998; Okamoto *et al*., 1998; Zondag *et al*., 1998), EDG3 (An *et al*., 1997; Okamoto, 1999), AGR16/EDG5 (Gonda *et al*., 1999), which indicates that sphingosine-1-phosphate works extracellularly as well as within the cell to evoke biological responses (Van Brocklyn *et al*., 1998). Notably, it has been shown that sphingosine-1-phosphate is abundantly stored in platelets (Yatomi *et al*., 1995a) and released upon platelet activation (Yatomi *et al*., 1995b). In terminally differentiated platelets, sphingosine-1-phosphate induces a shape change and aggregation by itself, and synergistically elicits aggregation in combination with weak platelet agonists such as epinephrine and adenosine diphosphate (ADP) (Yatomi *et al*., 1995b). Yatomi

et al. (1997) reported that sphingosine-1-phosphate is released from platelets into the serum during the blood clotting process, suggesting that sphingosine-1-phosphate release from activated platelets renders platelets to act as producers (from sphingosine) and releasers of sphingosine-1-phosphate *in vivo*. Additionally, sphingosine-1-phosphate has been shown to be involved in a variety of cellular functions including the stimulation of fibroblast growth (Zhang *et a*l., 1991; Olivera and Spiegel, 1993), regulation of cell motility (Sadahira *et al*., 1992; Bornfeldt *et al.*, 1995), activation of muscarinic K^+ currents in atrial myocytes (Bunemann *et al*., 1995; van Koppen *et al*., 1996), and suppression of ceramide-mediated apoptosis (Cuvillier *et al*., 1996).

In contrast to the well-characterized function of sphingosine-1-phosphate in platelets, very little is known about sphingosine in platelets. It might be possible that sphingosine is a metabolite very rapidly converted to either sphingosine-1-phosphate, ceramide, or that it is degraded.

Fig. 3:



(*from* Tani *et al*., *J Lip Res*, 2005)

Fig. 3: Scheme for the mechanism of sphingosine and sphingosine-1-phosphate generation in human platelets.

Cer: ceramide; SM: sphingomyelin; SMase: sphingomyelinase; Sph: sphingosine; S1P: sphingosine-1-phosphate (*from* Tani *et al*., *J Lip Res*, 2005).

1.3 Programmed Cell Death

1.3.1 Role of ceramide in apoptosis- the extrinsic pathway

Most studies on ceramide focused on cell death, which will be briefley discussed although cell death is not the primary focus of this discussion. The process of cell death involves programmed events regulated by specific biochemical pathways (Taha *et al*., 2006). Programmed cell death is driven by complex pathways involving several players, and can essentially be subdivided into three stages: a) intitation, b) commitment, and c) execution (Taha *et al*., 2006). The first step often occurs at or in the proximity of the cellular compartment where stress is induced (Feng and Hannun, 1998), like, for instance, disruption of Ca^{2+} homeostasis at the level of the endoplasmic reticulum, which leads to calcium-induced cell death (Breckenrisge *et al.*, 2003). This is turn causes the genotoxic stress in the nucleus to induce p53-driven cell death pathway activation (Miyashita and Reed, 2003; Oda *et al*., 2000; Yu *et al*., 2001; Nakano and Vousden, 2001). A further initiator of programmed cell death is the activation of cell death receptors at the level of the plasma membrane, involving the downstream activation of one or more organelle-mediated pro-apoptotic pathways (Jaattela, 2004; Barnhart *et al*., 2003). After biochemical pathways have been activated, triggered by a death signal, the cell converges onto the commitment step that may, in several instances, occur at the mitochondrial level (Ferri and Kroemer, 2001). Once the mitochondrion receives the death signal, a major mitochondrial change occurs, the mitochondrial membrane permeabilization, which, at the level of the outer mitochondrial membrane, causes the release of several proteins from the intermembrane space into the cytosolic space (Taha *et al*., 2006). Oligomerization of the adapter molecule APAF-1 is triggered by the release of cytochrome c,

causing the activation of caspase 9 (an integral cysteine protease in the mitochondrial cellular death pathway (Sellers and Fisher, 1999). Caspase 9 in turn activates caspases 3 and 7, members of the execution phase of programmed cell death that cleave proteins essential to normal cell function (Wolf and Green, 1999). A further protein that is released from mitochondria is Smac/Diablo (Taha *et al.*, 2006), which binds to inhibitors of apoptosis that usually function to keep caspases from activation. As cytochrome c and Smac are released, the cell shows typical features of apoptotic death, including chromatin condensation and externalization of phosphatidylserine.

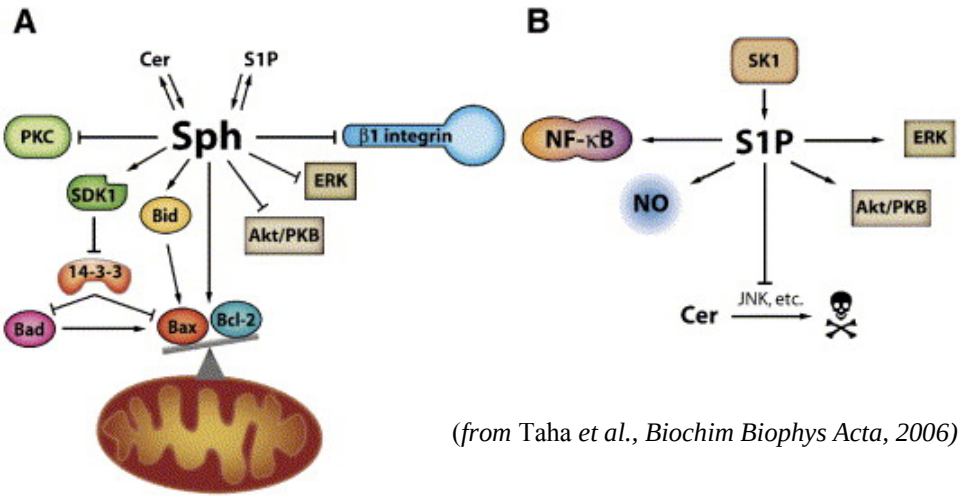
1.3.2 Role of mitochondria in apoptosis- the intrinsic pathway of apoptosis

Alternative forms of programmed cell death can be activated by release of different mitochondrial proteins that are caspase-independent. After mitochondrial membrane permeabilization, the proteins apoptosis-inducing factor (Gallego *et al*., 2004) and endonuclease G (Hahn *et al*., 2004) translocate from the mitochondrion to the nucleus, causing caspase-independent loose chromatin condensation (Taha *et al*., 2006). A further alternative form of cell death is comprised in the release of HtrA2/Omi, displaying a dual role in cell death in that the complex causes inhibitor of apoptosis inactivation, and displays serine protease activity (Hegde *et al*., 2002).

An entire family of proteins that is involved in the control of mitochondrial function in apoptosis is the members of the Bcl-2 family, which can be subdivided into three distinct classes: the Bcl-2-like survival factors like Bcl-2 and Bcl-x_L that maintain mitochondrial integrity (their overexpression guards the mitochondrion against stimuli normally inducing mitochondrial membrane permeabilization); the Bax-like death factors like Bax and Bad; and the BH3-only death factors such as Noxa, Puma, Bid, Bad, and Bik (Borner, 2003). The latter two subgroups compromise mitochondrial function and mediate cell death by several stimuli that induce mitochondrial membrane permeabilization, therefore indicating that the integrity of the cell and therefore the survival of the cell is dependent on the balance between the proapoptotic and the antiapoptotic Bcl-2 family members.

There are various reports showing the position of ceramide with respect to Bcl-2 proteins in that some groups have reported that ceramide acts upstream of Bcl-2 in the apoptotic pathway because of cell death rescue by Bcl-2 overexpression induced by ceramide (Zhang *et al*., 1996) or ceramidase inhibitors (Raisova, 2002). Also, it has been shown (El-Assaad *et al*., 1998) that Bcl-xL proteins rescue the cells from TNF-mediated cell death. This suggests that ceramide is involved in the cell death pathway downstream of Bcl-xL; however, other studies have shown that overexpression of Bcl-2 and Bcl-xL inhibits ceramide accumulation as a result of DNA damage stimulus (Tepper *et al*., 1999; El-Assaad *et al*., 1998; Sawada *et al*., 2000), implying that Bcl-2 can also act upstream of ceramide in the cell death pathway. Taha *et al*. (2006) suggested that not all ceramide species generate the same responses in regard to fatty acid chain length of ceramide, and that the different biological effects caused by ceramide may be mediated by distinct molecular species of the lipid (Taha *et al*., 2006).

Fig. 4 A+B:



(*from* Taha *et al., Biochim Biophys Acta, 2006)*

Fig. 4 A+B: Sphingosine and sphingosine-1-phosphate in apoptosis.

A: Cellular death is mediated through sphingosine by inhibiting the prosurvival factors PKC and Akt/PKB; cellular death is induced by SDK1, leading to activation of Bax by inhibiting the proteins 14-3-3. **B**: Apoptosis is inhibited by sphingosine-1-phosphate through activation of prosurvival factors like Akt/PKB, NF-κB, ERK and nitric oxide. Inactivation of JNK or other factors promoting programmed cellular death may also inhibit ceramide-induced cell death by sphingosine-1-phosphate. (*from* Taha *et al., Biochim Biophys Acta, 2006)*

Experiments by Gudz *et al*. (1997) have revealed that several changes in isolated mitochondria can be induced by ceramide *in vitro* (Gudz *et al*., 1997). C2-ceramide inhibited oxidative phosphorylation by electron transport chain interference at the level of complex III, and ceramide also increases the permeabiliy of the outer mitochondrial membrane to several proteins including cytochrome c (Siskind *et al*., 2002). Furthermore, Novgorodov *et al*. (2006) demonstrated that ceramide is able to enhance the permeability of the inner mitochondrial membrane by action of the permeability transistion pore and the electrogenic proton channel, enhancing the release of solutes into the cytosol (Novgorodov *et al*., 2006). It was shown by Lorusso *et al.* and Di Paola *et al*., 2004) that ceramide induces cytochrome c release from isolated mitochondria in a permeability transition pore-dependent and independent manner, and Ghafourifar *et al*. (1999) showed that ceramide maintains cytochrome c in an oxidized state which seems to be necessary for reducing mitochondrial oxygen consumption and releasing calcium from mitochondria. Therefore, it appears that there is a direct effect of ceramide on mitochondria. Further investigation into sphingomyelin hydrolysis specific to organelles has revealed that only ceramide generation at the mitochondrial level can cause cell death, while its formation in other organelles fails to do so (Birbes *et al*., 2001).

1.4 Aims of the Study

The present study will aim to identify the function of acid sphingomyelinase in platelets as crucial for for hematogenous metastasis of melanoma cells into the lung. The interaction of tumor cells with platelets and a possible activation of the enzyme acid sphingomyelinase that results in a release of ceramide from platelets was studied. Furthermore, knock-out mice deficient for acid sphingomyelinase were employed to define the biological function of the acid sphingomyelinase for tumor metastasis *in vivo*.

Furthermore, this study aims to show whether activation of acid sphingomyelinase by melanoma cells is mediated by the P-selectin molecule present on platelets and abrogated in platelets deficient for the P-selectin molecule. Studies were carried

out to determine whether P-selectin-deficient platelets still interact with tumor cells. To this end, tyrosine phosphorylation induction of several cellular substrates was investigated in both, wild-type and P-selectin-deficient platelets. To determine whether P-selectin activation occurs upstream of acid sphingomyelinase activation or vice versa, platelet degranulation and P-selectin/GPIIβIIIα expression after stimulation with B16F10 melanoma cells will be investigated in wild-type and ASM-deficient platelets; analysis thereof will also indicate whether P-selectin itself prevents activation of thrombocytes by B16F10 melanoma cells. Finally, it was determined whether fucoidan, a natural ligand of P-selectin, is able to prevent melanoma metastasis in wild-type mice.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Enzymes

Unless otherwise noted, all chemicals and enzymes were purchased from Sigma, Roth, Gibco, Serva, Fluka, J.T. Baker, Amersham, Invitrogen, Perkin-Elmer or Merck. Radioactive compounds were purchased from Perkin-Elmer or Hartmann Analytics. The following table of chemicals including radioisotopes and enzymes indicates the name of the chemical and the source from where it was obtained.

Chemical Name	**Source**
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim
Acetic Acid	Merck, Darmstadt
Agarose	Gibco/Invitrogen, Karlsruhe
Albumin Fraction V, Fatty-Acid free	Carl Roth GmbH&Co., Karlsruhe
Aprotinin	Sigma-Aldrich Chemie GmbH, Steinheim
ATP	Sigma-Aldrich Chemie GmbH, Steinheim
Boric Acid	Sigma-Aldrich Chemie GmbH, Steinheim
Bromophenol Blue	Serva Electrophoresis GmbH, Heidelberg
C16 Ceramide	BioMol, PA, USA
Calcium Chloride	Sigma-Aldrich Chemie GmbH, Steinheim
Cardiolipin	Sigma-Aldrich Chemie GmbH, Steinheim
CDP-Star	Tropix
Chloroform	Riedel-de-Haen, Seelze
DAG Kinase	Amersham
Diethylenetriaminepentaacetic acid, DETAPAC	Sigma-Aldrich Chemie GmbH, Steinheim
Dithiothreitol, DTT	Carl Roth GmbH&Co., Karlsruhe
EGTA	Carl Roth GmbH&Co., Karlsruhe
Ethanol	Fluka Chemie GmbH, Buchs
Ethidium Bromide	Serva Electrophoresis GmbH, Heidelberg
Ethylenediaminetetraacetic acid, EDTA	Serva Electrophoresis GmbH, Heidelberg
Fucoidan	Sigma-Aldrich Chemie GmbH, Steinheim
Glucose	Sigma-Aldrich Chemie GmbH, Steinheim
Glycine	Carl Roth GmbH&Co., Karlsruhe
Hydrochloric Acid	J.T. Baker

Imidazole-HCl	Sigma-Aldrich Chemie GmbH, Steinheim
Isopropanol	J.T. Baker
Leupeptin	Sigma-Aldrich Chemie GmbH, Steinheim
Magnesium Chloride	Sigma-Aldrich Chemie GmbH, Steinheim
Magnesium Sulfate	Sigma-Aldrich Chemie GmbH, Steinheim
Methanol	Fluka Chemie GmbH, Buchs
n-Octylglucopyranoside	Sigma-Aldrich Chemie GmbH, Steinheim
NP-40	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium Chloride	Sigma-Aldrich Chemie GmbH, Steinheim
Proteinase K	Sigma-Aldrich Chemie GmbH, Steinheim
Scintillation Liquid	Beckman-Coulter, Fullerton, CA, USA
Sodium Acetate	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium Carbonate	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium Chloride	Carl Roth GmbH&Co., Karlsruhe
Sodium Citrate	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium Deoxycholate	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium Dodecyl Sulfate, SDS	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium Fluoride	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium Orthovanadate	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium Phosphate	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium Pyrophosphate	Sigma-Aldrich Chemie GmbH, Steinheim
Tris-HCl	Carl Roth GmbH&Co., Karlsruhe
Triton X-100	Sigma-Aldrich Chemie GmbH, Steinheim
Tween	Sigma-Aldrich Chemie GmbH, Steinheim

2.1.2 Tissue Culture Materials

Dissociation Buffer, Enzyme-free	Gibco/Invitrogen, Karlsruhe
Fetal Bovine Serum, FBS	Gibco/Invitrogen, Karlsruhe
HEPES	Carl Roth GmbH&Co., Karlsruhe
L-Glutamine	Gibco/Invitrogen, Karlsruhe
MEM Medium	Gibco/Invitrogen, Karlsruhe
Non-Essential Amino Acids	Gibco/Invitrogen, Karlsruhe
Penicillin	Gibco/Invitrogen, Karlsruhe
Sodium Pyruvate	Gibco/Invitrogen, Karlsruhe
Streptomycin	Gibco/Invitrogen, Karlsruhe
T75 Tissue Culture Flasks	TPP, Trasadingen, Switzerland
Trypsin	Gibco/Invitrogen, Karlsruhe

2.1.3 Radioactive Compounds

[^{32}P]- γ- ATP	Hartmann Analytic, Braunschweig
[^{14}C]- Sphingomyelin	Perkin-Elmer, Boston, MA, USA

2.1.4 Common Solutions used

10x TBS-Tween	200 mM Tris-Cl pH 7.4 1.5 M NaCl 1% Tween
Protein Transfer Buffer	25 mM Tris 192 mM Glycine 20% Methanol
Tissue Lysis Buffer	1x PCR buffer (Solis) 0.5 mM $MgCl_2$ 0.045% Tween-20 0.045% IGEPAL 300 µg/ml Proteinase K
10x HEPES-Saline	200 mM HEPES 1.32 M NaCl 10 mM $CaCl_2$ 7 mM $MgCl_2$ 8 mM $MgSO_4$ 54 mM KCl
1x TBE	90 mM Tris-Acetate pH 7.8 10 mM Boric Acid 1 mM EDTA
1x PBS	137 mM NaCl 2.6 mM KCl 6.5 mM Na_2HPO_4 1.5 mM KH_2PO_4

Ethidium Bromide	10 mg/ml Ethidium Bromide in H_2O
5x SDS Sample Buffer	250 mM Tris pH 6.8 20% Glycine 4% SDS 8% 2-Mercaptoethanol 0.2% Bromophenol blue
6x DNA Loading Buffer	50% Glycerol 0.02% Bromophenol blue 0.04% Xylene Cyanol 1mM EDTA
SDS-PAGE	Solution A: 40% Acrylamide (39.2g Acrylamide /100 ml; 0.8 g Bis-Acrylamide /100 ml) Solution B: 3M Tris pH 8.8 Solution C: 3M Tris pH 6.8
Tyrode Buffer	20 mM HEPES pH 7.4 134 mM NaCl 2.9 mM KCl 12 mM $NaHCO_3$ 1 mM $CaCl_2$ 5 mM Glucose
Alkaline Wash Buffer	100 mM Tris pH 9.5 100 mM NaCl

2.1.5 Oligonucleotide Primers used for Genotyping

The utilized ASM DNA oligonucleotides were purchased from Hermann GbR Freiburg, the oligonucleotides for mycoplasma were from MWG Biotech.

ASM-PA1-2: 5'- CGAGACTGTTGCCAGACATC -3'

ASM-PA2-2: 5'- GGCTACCCGTGATATTGCTG -3'

ASM-PS-2: 5'- AGCCGTGTCCTCTTCCTTAC -3'

Mycoplasma sense: 5'- GTGCCAGCAGCCGCGGTAATA -3'

Mycoplasma antisense: 5'- TACCTTGTTACGACTTCACCCCA -3'

2.1.6 Antibodies

For Western Blot protein analysis the following primary and secondary antibodies were utilized:

Primary Antibodies

PE- conjugated rat-anti-mouse Glycoprotein αIIbβ3 (emfret analytics)
FITC- conjugated rat-anti-mouse P-Selectin CD62P (emfret analytics)
Phospho-P44/42 MAP Kinase (T202/Y204) E10 Monoclonal antibody (Cell Signaling)
Phospho- SAPK/JNK (Thr183/Tyr185) Antibody (Cell Signaling)
Anti-Phosphotyrosine Clone 4G10 Mouse Monoclonal IgG Antibody (Upstate)
Anti-PLCγ-1 Clone B-6-4 Mouse Monoclonal IgG Antibody (Upstate)

Secondary Antibodies

All secondary antibodies were purchased from Santa Cruz

Anti-Goat IgG-Alkaline Phosphatase-coupled
Goat-anti-Rabbit IgG Alkaline Phosphatase-coupled
Goat-anti-Guinea Pig IgG Alkaline Phosphatase-coupled
Goat-anti-Mouse IgG Alkaline Phosphatase-coupled

For flow cytometry experiments the above mentioned antibodies

PE- conjugated rat-anti-mouse Glycoprotein αIIbβ3 and
FITC- conjugated rat-anti-mouse P-Selectin CD62P

were used.

2.2 Organisms

Wild-type C57/Bl6 mice and P-Selectin-deficient mice were obtained from Jackson Laboratories; the ASM-deficient mice were provided by Dr. Richard Kolesnick (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). Mice were housed and bred in the Central Animal Facility at the University of Duisburg-Essen Medical College, Essen, Germany. Mice were fed standard-type pellet food, water was available *ad libitum*, and the mice were housed in autoclaved cages. The mice were monitored daily for any signs of illness or disease. Mice were housed in a 12-hour light cycle.

2.3 Methods

2.3.1 Methods in Molecular Biology

2.3.1.1 DNA Techniques

2.3.1.1.1 DNA Isolation from Mouse Tails

For the isolation of DNA from mouse tails for purpose of genotyoing, app. 1-2 mm of mouse tail was cut and placed into 80 ul tissue lysis buffer containing Proteinase K. The samples were incubated at 56°C overnight and the volumes were raised to 800 µl with autoclaved ddH_2O. For PCR analysis, 1 µl of the lysate was used.

2.3.1.1.2 Polymerase Chain Reaction

For the identification of ASM wild-type, ASM heterozygotes (+/-) or ASM knock-out (-/-) mice the polymerase chain reaction was utilized. For this reaction, 1 µl of the overnight tail digest (lysed in 80 µl lysis buffer and raised to 800 µl in ddH_2O) was added to 1.2 µl 10x PCR buffer, 2.5 mM $MgCl_2$, 1 µl dNTP mix (2.5 mM) and 10 pmol each of primers ASM-PA1-2, ASM-PA2-2 and ASM-PS-2 in a thin-walled 0.2-ml-PCR tube. The temperature of the lid of the PCR Machine was raised to 104°C, and the temperature of the PCR block was raised to 96°C for 17 min, after which the follwing cycle was carried out 35 times:

Denaturation: 95°C for 1 min.
Annealing: 58°C for 1 min.
Elongation: 72°C for 1 min., 45 sec.

After the last cycle the PCR block remained at 72°C for 5 minutes to complete elongation reactions, after which the samples were placed at 4°C. 5 µl of each sample was loaded onto an electrophoretic gel for genotype determination.

Cells in culture were regularly tested for the presence of contamination by mycoplasma. For this, app. $1x10^5$ B16F10 cells were counted and pelleted by centrifugation. 50 µl of tissue lysis buffer were added and the sample was incubated at 56°C for app. 3 hours, after which the samples were boiled for 10 minutes. The samples were diluted with 50 µl ddH_2O, and for DNA quantification 10 µl of the sample was further diluted 1:50 with ddH_2O and placed in a glass cuvette for measurement of absorption at 260 nm wavelength.

For the polymerase chain reaction, 1 µl of the DNA was added to 2.5 µl 10x PCR buffer (Solis), 1.5 µl 25 mM $MgCl_2$, 0.5 µl of 10mM dNTPs, 0.25 µl of both mycoplasma primers, and 1.25 U Taq polymerase (Solis). The volume was raised to 25 µl with ddH_2O in a 0.2 ml thin-walled PCR reaction tube. The samples were heated to 96°C for 17 minutes and the following cycle was repeated 25 times:

Denaturation:	95°C for 1 minute
Annealing:	60°C for 1 minute
Elongation:	72°C for 1.5 minutes

After the cycles were carried out, the PCR block remained at 72°C for a further 7 minutes to complete elongation. The samples (15 µl of the reaction) were loaded onto a 1% agarose gel.

2.3.1.1.3 Visualization of Amplified Products

In order to determine the ASM genotype of mice, a 1% (w/v) electrophoretic agarose gel was poured in 1x TBE buffer that contained 20 µg ethidium bromide. The PCR product samples (15 µl) were applied to the wells of the gel under 1x TBE along with a 100-bp-standard, and a current of 5V/cm between anode and cathode was applied. Visualization of the DNA fragments took place by UV light at 302 nm wavelength due to the intercalation of Ethidium Bromide in the DNA.

2.3.1.2 Protein Techniques

2.3.1.2.1 Production of Whole-Cell Lysates

Thrombocytes were stimulated with B16F10 melanoma cells for indicated times and immediately lysed on ice with lysis buffer as described above. The samples were boiled for 5 min in 1x SDS loading dye containing disulfide bond-denaturing 2-mercaptoethanol.

2.3.1.2.2 Concentration Determination of Proteins

To determine the amount and concentration of proteins in whole-cell lysates, 10 µl of the sample was analyzed spectrophotometrically at a wavelength of 595 nm. Known amounts of BSA were analyzed simultaneously for production of a standard curve. For analysis, proteins of either standard or unknown sample were placed in 1 ml of commercially available Bradford solution (BioRad).

2.3.1.2.3 Separation of Proteins on SDS-PAGE

Equal amounts of each sample were applied to the wells of 7.5%, 10% or 12% SDS PAGE gels. The composition of the gels was as follows:

Running gel: 7.5% SDS PAGE: 2.81 ml Solution A
5.0 ml Solution B
7.05 ml H_2O
0.075 ml 20% SDS
0.03 ml N,N,N',N'-Tetramethylenediamine, TEMED
0.03 ml 20% Ammonium persulfate, APS (BioRad)

10% SDS-PAGE:	3.75 ml Solution A
	5.0 ml Solution B
	6.11 ml H_2O
	0.075 20% SDS
	0.03 ml TEMED
	0.03 ml 10% APS
12% SDS-PAGE:	4.5 ml Solution A
	5.0 ml Solution B
	5.36 ml H_2O
	0.075 ml 20% SDS
	0.03 ml TEMED
	0.03 ml 10% APS
Stacking Gel:	0.63 ml Solution A
	0.21 ml Solution C
	4.1 ml H_2O
	0.025 ml 20% SDS
	0.02 ml TEMED
	0.02 ml 10% APS

The protein samples were applied to the gel and run at 25 V per gel through the stacking gel, after which the voltage was raised to 50V per gel as the proteins were carried through the separating gel. A standard that contains a bromophenol blue dye was run along with the samples for later size identification. Once the dye reached to bottom of the gel, the apparatus was dismantled and the gel was briefly soaked in transfer buffer. The gel was placed upon a nitrocellulose membrane (Hybond ECL), and the proteins were transferred onto the membrane in a BioRad Tank Blot Mini Protean II cassette. After washing of the membrane with TBS-T and exposure to primary and secondary antibody, an X-Ray film (Amersham Biosciences) was exposed for various times to show protein bands.

2.3.1.2.4 Tyrosine- and Signaling Molecule Phosphorylation

Platelets were stimulated by melanoma cells for the indicated stimulation times, lysed on ice and centrifuged at 15,000 rpm for 5 minutes to pellet nuclei and insoluble cellular debris. Supernatants received 5 x reducing SDS sample buffer containing 250 mM Tris-HCl pH 6.8, 20 % Glycine, 4 % SDS, 8 % 2-Mercaptoethanol and 0.2 % Bromophenol blue and were boiled for 5 minutes. Samples were added to sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) ranging from 7-12 % and electrophoresed and blotted onto nitrocellulose membranes. Membranes received 4 % BSA in Tris-buffered saline (TBS) for blocking for 1 h, washed in TBS supplemented with 0.1 % Tween (TBS-T) and exposed to 1 μg/ml monoclonal anti-phosphotyrosine 4G10 (UBI), anti- phospho-PLCγ (phospho-Tyr 1217, NEB), anti- phospho- MAP-K (phospho- Thr 202/ phospho- Tyr 204, NEB) or anti- phospho- JNK (phospho- Thr 183/ phospho- Tyr 185, NEB) antibodies for 4 hours at room temperature or overnight at 4°C. After exposure, the blots were extensively washed with TBS-T and subjected to alkaline phosphatase- coupled secondary antibodies at a concentration of 1:20,000 in 20 ml TBS-T for 60 minutes at room temperature. After further extensive washing, the blots were developed using the Tropix chemilluminescence system after brief exposure of the blots to alkaline wash buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) and exposed to X-Ray film.

2.3.2 Cell Culture Techniques

2.3.2.1 Passage of Cell Cultures

B16F10 malignant melanoma cells were cultured in MEM supplemented with 10% Fetal Bovine Serum, 10 mM HEPES pH 7.4, 2 mM L-Glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 100 units/ml penicillin and 100 ug/ml streptomycin (all from Gibco). The cells were replaced by a freshly thawed and cultured aliquot after 21 days of growth to avoid selection of specific clones. Cells were grown to subconfluency prior to all experiments. For passage, the cells were washed once in PBS and released from the T75 flask by addition of 0.5% Trypsin and incubation for 5 minutes at 37°C.

Trypsinization reaction was stopped by addition of 5 volumes supplemented MEM medium.

2.3.2.2 Freezing and Thawing of Cells

For long-term storage of cell lines the cells were centrifuged for 5 minutes at 250xg and resuspended in 1 ml freezing medium consisting of 70% supplemented medium, 20% FBS and 10% DMSO and transferred to a freezing vial (Nunc) and frozen at –80°C. After 24 hours the vials were placed into liquid nitrogen. Thawing of cells occurred by placement of the vials into a 37°C water bath. The cells were once washed in 10 ml of supplemented medium to get rid of DMSO, and placed into T75 flasks.

2.3.2.3 B16F10 Melanoma Cell Purification

Malignant melanoma cells (B16F10) were cultured in supplemented Mulbecco Eagle Medium (MEM) (see above). Cells were grown to subconfluency and released from the T75 flask by use of 3 ml cell dissociation buffer (Invitrogen). Melanoma cells were washed three times in 10 ml 1 M HEPES-Saline pH 7.4 (132 mM NaCl, 20 mM HEPES pH 7.4, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$) with centrifugation steps at 250 x g for 5 minutes at room temperature. The tumor cells were counted and resuspended in 20 µl 1x HEPES- Saline and equilibrated at 37°C for 8 minutes prior to use in stimulation experiments.

2.3.3 Mouse Thrombocyte Isolation

Mice were either bled retroorbitally or bled by a cut in the tail vein. Approximately 500 µl blood was obtained and immediately mixed with 3.8 % sodium citrate in a 1.5-ml-Eppendorff tube. After dilution of the blood with 10 ml of 3.5 % Albumin (Sigma; Fraction V; fatty-acid free) the samples were incubated at 37°C for 15 minutes and centrifuged at 100 x g for 20 minutes without brake at room temperature. The supernatants

were collected and re-centrifuged at 1340 x g for 15 minutes at room temperature to pellet thrombocytes. The platelets were resuspended in Tyrode buffer (20 mM HEPES at pH 7.4, 134 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$ and 5 mM glucose), counted and equilibrated at 37°C for 8 minutes before stimulation experiments.

2.3.4 *In Vivo* Metastasis

Malignant melanoma cells (B16F10) were released from T75 flasks by washing the cells in PBS and addition of 3 ml enzyme-free cell dissociation buffer (Gibco). Cells were washed extensively and resuspended in 200 µl 1 x HEPES- Saline. Mice were injected intravenously via the retrobulbar eye plexus with 100,000 melanoma cells. After 21 days the mice were sacrificed and the visible metastases in the lung were counted.

2.3.5 Interaction of Platelets with Tumor Cells

2.3.5.1 Time Course Experimental Setup

Tumor cells were released from T75 flasks by Cell Dissociation Buffer (Enzyme-free, PBS-based; Gibco) and washed extensively. After resuspension in 1 x HEPES-Saline the cells were equilibrated at 37°C for 8 minutes and added to platelets at a ratio of 1 tumor cell per 100 platelets to initiate stimulation. Platelets were allowed to be stimulated for 0 sec (control), 15 sec., 30 sec. 45 sec., 60 sec., and 90 sec. Termination of stimulation was achieved by addition of cell lysis buffer (25 mM HEPES pH 7.4, 0.1 % sodium dodecyl sulfate (SDS), 0.5 % sodium deoxycholate, 1 % Triton X-100, 100 mM NaCl, 10 mM NaF, 10 mM $Na_2P_2O_7$, 10 mM Na_3VO_4, 10 mM ethylene diamine tetraacetic acid (EDTA) and 10 ug/ml aprotinin and 10 ug/ml leupeptin) on ice for 5 minutes. Control experiments were treated with equal volumes of HEPES-Saline.

2.3.5.2 Tyrosine- and Signaling Molecule Phosphorylation

Platelets were stimulated by melanoma cells for the indicated stimulation times, lysed on ice and centrifuged at 15,000 rpm for 5 minutes to pellet nuclei and insoluble cellular debris. Supernatants received 5 x reducing SDS sample buffer containing 250 mM Tris pH 6.8, 20 % Glycine, 4 % SDS, 8 % 2-Mercaptoethanol and 0.2 % Bromphenol blue and were boiled for 5 minutes. Samples were applied to sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) ranging from 7-12 % (for detection of Phosphotyrosine, a 12 % gel was used, as well as for detection of MAP kinase and JNK; detection of PLCγ required a 7 % gel) and and electrophorettically blotted onto nitrocellulose membranes. Membranes received 4 % BSA in Tris-buffered saline (TBS) for blocking for 1 h, washed in TBS supplemented with 0.1 % Tween (TBS-T) and exposed to 1 μg/ml monoclonal anti-phosphotyrosine 4G10 (UBI), anti- phospho-PLCγ (phospho-Tyr 1217, NEB), anti-phospho- MAP-K (phospho- Thr 202/ phospho- Tyr 204, NEB) or anti- phospho- JNK (phospho- Thr 183/ phospho- Tyr 185, NEB) antibodies for 4 hours at room temperature or overnight at 4°C. After exposure, the blots were extensively washed with TBS-T and subjected to alkaline phosphatase- coupled secondary antibodies for 60 minutes at room temperature. After further extensive washing, the blots were developed using the Tropix chemilluminescence system after brief exposure of the blots to alkaline wash buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5).

2.3.6 Ceramide Release Assay

After platelet stimulation by B16F10 tumor cells and cell lysis, the platelets were extracted with $CHCl_3$:CH_3OH:1N HCl (100:100:1; v/v/v). The organic phase was obtained and dried using a speedvac. Diacylglycerol was degraded in 0.1 N methanolic KOH at 37°C for 60 minutes. After re-extraction the organic phase was dried and resuspended in 20 μl of a detergent solution (7.5 % (w/v) n-octylglucopyranoside, 5 mM cardiolipin, 1 mM diethylenetriaminepentaacedic acid, DETAPAC). Formation of micelles was initiated by bath sonication for 10 minutes. A phosphorylation buffer (0.1 M imidazole/HCl pH 6.6, 0.1 M NaCl, 25 mM $MgCl_2$, 2 mM EGTA, 2.8 mM dithiothreitol (DTT), 1 μM adenosine triphosphate (ATP), 10 μCi [^{32}P]-γ ATP and 0.024 units/sample DAG kinase)

initiated ceramide phosphorylation, and the samples were incubated at room temperature for 30 minutes. Termination of the reaction was achieved by addition of 1 ml $CHCl_3$:CH_3OH:1N HCl (100:100:1; v/v/v), 170 µl buffered saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM Glucose, 10 mM HEPES pH 7.2) and 30 µl of a 100 mM EDTA solution. The resulting upper phase was removed, and the lower organic phase was again concentrated by SpeedVac centrifugation. The dried lipids were dissolved in 20 µl/sample $CHCl_3$:CH_3OH (1:1 v/v). Lipids were separated on a Silica G60 TLC plate. A solvent system of $CHCl_3$:CH_3OH:CH_3COOH (65:15:5 v/v), was added to the TLC chamber, and was allowed to saturate the atmosphere for 1 h by using a sheet of Whatman filter paper. The silica plates were loaded with the solubilized lipids, placed into the TLC chamber and the solvent front was allowed to migrate to the top of the plate. The plate was then removed, air dried for 45 min and exposed to X-ray films for 24-72 hrs. Ceramide-spots were identified by co-migration with a C_{16}-ceramide-1-phosphate standard, scraped from the plate into 20 ml polyethylene vials and quantified by liquid scintillation counting using the Cerenkov protocol.

2.3.7 Acid Sphingomyelinase Assay

After stimulation of platelets with B16F10 melanoma cells, the cells were lysed with 250 mM sodium acetate pH 5.0, 1.3 mM EDTA and 1% NP-40. After three sonication steps for 10 seconds each, the samples were incubated for 30 minutes at 37°C with [^{14}C]-Sphingomyelin (0.05 µCi/sample, corresponding to 52 mCi/mmol; MP-Biomedicals) that was resuspended in 250 mM sodium acetate pH 5.0, 1.3 mM EDTA and 0.1% NP-40 after drying. Termination of the sphingomyelinase reaction was achieved by extraction in 8 vol of $CHCl_3$:CH_3OH (2:1, v/v). The phases were separated by centrifugation and the upper phase was placed into liquid scintillation fluid for radioactive scintillation counting. As measurement for the activity of the enzyme, hydrolysis of [^{14}C]-Sphingomyelin by the acid spingomyelinase results in the formation of water-soluble [^{14}C]-Choline chloride that is extracted in the upper phase. [^{14}C]-Sphingomyelin was first dried by SpeedVac centrifugation and solubilized into micelles in ASM-lysis buffer, using a bath sonicator for 10 min. Lipids were extracted by addition of 1 ml/sample chloroform:methanol (2:1, v/v), followed by vigorous vortexing for 30 sec and centrifugation at 800 x g for 10 min. This

results in separation of phosphorylcholine in the upper (aqueous) phase and ceramide in the lower (organic) phase. An aliquot (350 µl) of the upper phase was carefully collected and transferred in 20 ml polyethylene vials filled with 3.5 ml Scintillation Cocktail, followed by quantification by liquid scintillation counting.

2.3.8 Fucoidan Experiments

2.3.8.1 Fucoidan Preparation and Mouse Injection

Fucoidan stock was prepared at a concentration of 10 mg/ml in HEPES-Saline pH 7.4 and sterile-filtered. Unused aliquots were frozen at –20°C. For injection, fucoidan was diluted in HEPES-Saline. Mice were injected buffer only as control (HEPES-Saline), 0.025 µg, 0.1 µg, 0.4 µg and 2 µg in a total volume of 200 µl. Injection occurred through the tail vein or the retrobulbar complex.

2.3.8.2 B16F10 Mouse Injection

B16F10 melanoma cells were released from T75 flasks and purified as described above. The cells were resuspended in 200 µl HEPES-Saline to yield 100,000 cells per injection. Mice were injected via the retrobulbar eye complex approximately 2 hours after fucoidan injection. Control mice received 200 µl HEPES-Saline only.

2.3.8.3 Mouse sacrifice and lung metastasis determination

21 days after fucoidan and B16F10 melanoma cell injection, the mice were sacrificed by cervical dislocation and the lungs removed. Visible metastases were counted and recorded.

2.3.8.4 FACS Analysis of Platelet Degranulation and P-selectin Expression

Mouse thrombocytes were isolated as described above (see **2.3.3**). B16F10 melanoma cells were released from T75 flasks by Cell Dissociation Buffer (Enzyme-free, PBS-based; Gibco), washed extensively in HEPES-Saline, and allowed to remain at 37°C. After resuspension in 1 x HEPES-Saline the thrombocytes were equilibrated at 37°C for 5 minutes and added to 2 mg/ml fucoidan in HEPES-Saline. After a further 3 minutes at 37°C tumor cells were added to the platelet:fucoidan mix at a ratio of 1 tumor cell per 100 platelets to initiate stimulation. Anti-P-selectin antibody was added immediately at a concentration according to the manufacturer's protocol (Emfret), and the reactions were allowed to remain at room temperature for 15 minutes. The volume was raised to 500 µl with phosphate-buffered saline (PBS; without Mg^{2+}, Ca^{2+}). FACS analysis was performed.

3. Results

3.1 Metastasis of Melanoma Cells in Acid Sphingomyelinase-Deficient and Wild-Type Mice

3.1.1 Acid sphingomyelinase deficiency attenuates B16F10 melanoma metastasis

Acid Sphingomyelinase-positive and -deficient mice were injected intravenously with 1 x 10^5 B16F10 melanoma cells in order to investigate the function of sphingolipids and acid sphingomyelinase for platelet-mediated tumor metastasis. After 21 days these mice were sacrificed by cervical dislocation, the lungs were removed, and the number of visible lung metastases was determined. Results revealed a vast difference between hematogenous tumor cell metastasis in wild type mice with 54 ± 5 pulmonary metastases compared to acid sphingomyelinase-deficient mice with 1.8 ± 1.9 pulmonary metastases, indicating a reduction of pulmonary B16F10 melanoma cell metasases of 97% in the lungs of acid sphingomyelinase-deficient mice. These data depicted in Fig. 5 A and 5 B suggest a pivotal role of the acid sphingomyelinase for the metastasis of B16F10 melanoma cells to the lung.

Fig. 5 A:

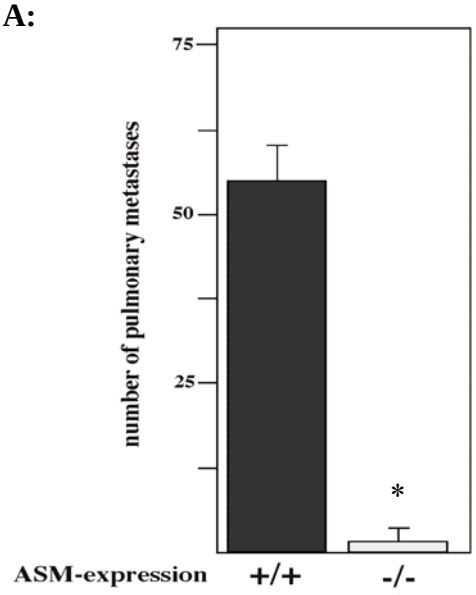


Fig. 5 B:

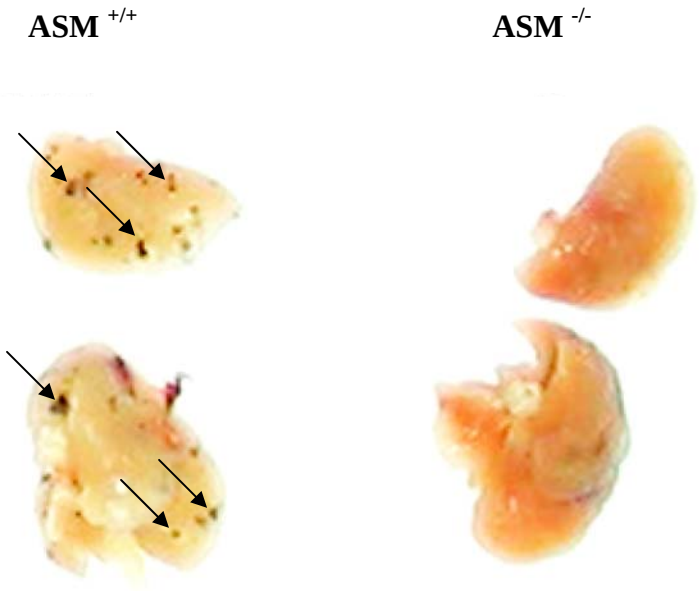


Fig. 5 A + B: Metastasis of melanoma cells is prevented in acid sphingomyelinase-deficient mice

Acid sphingomyelinase-positive and -deficient mice were intravenously injected with $5x10^4$ B16F10 melanoma cells, and the number of lung metastases was determined after 21 days. Deficiency of acid sphingomyelinase prevented metastasis of B16F10 tumor cells into the lung, which occurred at multiple sites in wild-type mice. Panel **A** shows the mean ± SD of the number of pulmonary B16F10 metastases in 9 wild-type and 8 acid sphingomyelinase-deficient mice, respectively. Significant differences ($p \leq 0.05$, *t*-test) between ASM-positive and ASM-deficient cells are indicated by an asterisk*. Panel **B** displays the lungs of a typical experiment. The dark spots in the lung tissue on the left-side panel are the melanoma metastases (arrows) that are absent in the lungs on the right-hand panel.

3.2 Interaction of Platelets with B16F10 melanoma cells

3.2.1 Tumor cells induce activation of acid sphingomyelinase in platelets

In order to determine whether the interaction of platelets with B16F10 tumor cells causes stimulation of the acid sphingomyelinase, platelets of wild-type and acid sphingomyelinase-deficient mice were purified and co-incubated with B16F10 tumor cells and the activity of the acid sphingomyelinase was determined. Results (n=4) show a rapid stimulation of the acid sphingomyelinase upon co-incubation of wild-type platelets with

tumor cells, as shown in Fig. 6 A. Acid sphingomyelinase activity increased within 5 seconds of co-incubation with tumor cells, peaked at 10 seconds and declined to baseline within 45 seconds. When platelets of acid sphingomyelinase-deficient mice were co-incubated with tumor cells, no increase of acid sphingomyelinase activity was observed (Fig. 6 B), indicating that tumor-platelet interaction results in an exclusive activation of the platelet acid sphingomyelinase, while the enzyme acid sphingomyelinase present in the B16F10 tumor cells does not seem to be activated upon contact with platelets.

Fig. 6 A:

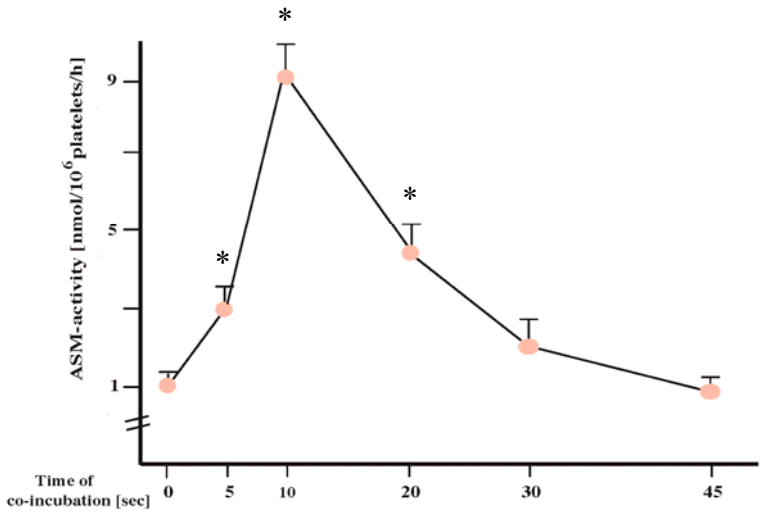


Fig. 6 B:

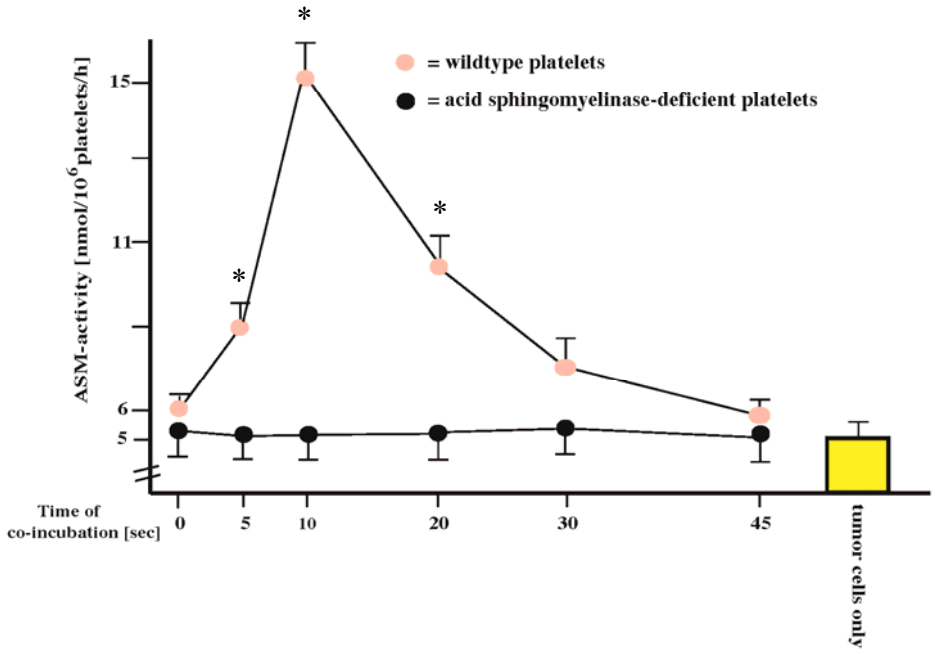


Fig. 6 A+B: Tumor cells induce activation of acid sphingomyelinase in platelets.

(**A** and **B**) Incubation of wild-type platelets with B16F10 tumor cells induced a rapid, transient activation of acid sphingomyelinase (ASM). No changes of the enzyme's activity were observed upon co-incubation of acid sphingomyelinase-deficient platelets with tumor cells indicating an activation of the platelet acid sphingomyelinase, while the activity of the enzyme in tumor cells seems to be unaffected. Panel **A** displays the activity of the acid sphingomyelinase in wildtype platelets after subtraction of the acid sphingomyelinase activity in tumor cells. Significant differences ($p \leq 0.05$, *t*-test) between ASM activity of stimulated vs. unstimulated cells are indicated by an asterisk*. Panel **B** demonstrates the total acid sphingomyelinase-activity after co-incubation of B16F10 cells with platelets. Total activity of the unstimulated samples was achieved by addition of the activity in untreated platelets and tumor cells. Platelets were purified from wild-type and acid sphingomyelinase-deficient mice, stimulated with tumor cells (10^5 tumor cells/10^7 platelets), lysed and the acid sphingomyelinase activity was determined by the consumption of [^{14}C]-sphingomyelin added to the lysates. Displayed is the mean ± SD of 4 independent experiments. Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated samples are indicated by an asterisk*.

3.2.2 Tumor cells induce the release of ceramide from platelets

Since acid sphingmyelinase hydrolyzes sphingomyelin to form ceramide, stimulation of acid sphingomyelinase in platelets isolated from wild-type mice with B16F10 tumor cells resulted in a release of ceramide, while purified platelets isolated from acid sphingomyelinase-deficient mice failed to release ceramide upon stimulation with B16F10

melanoma cells. These data shown in Fig. 7 indicate that acid sphingomyelinase in platelets and not in the tumor cells mediated the release of ceramide upon co-stimulation of the cells.

Fig. 7

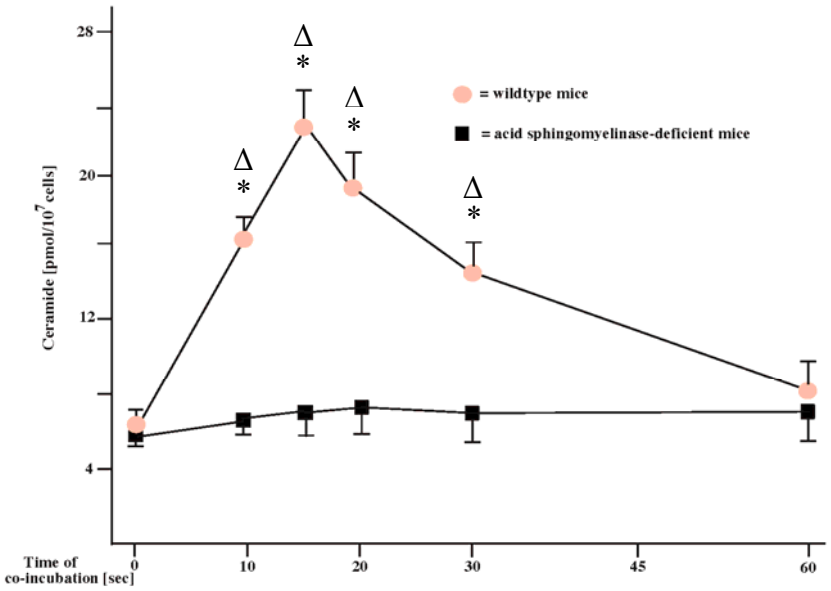


Fig. 7: Ceramide is rapidly released upon co-incubation of wild-type platelets with B16F10 tumor cells.

When platelets were co-incubated with B16F10 melanoma cells, release of ceramide was observed within 10 seconds of co-incubation, while no release of ceramide was observed in acid sphingomyelinase-deficient platelets stimulated with the tumor cells. Platelets were purified from wild-type and acid sphingomyelinase-deficient mice, co-incubated with tumor cells as above and ceramide release was determined by the DAG-kinase assay. Ceramide in tumor cells was subtracted from the total amount of ceramide in the samples to calculate the formation of ceramide in the platelets. Displayed are the means ± SD of 3 independent experiments.

Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated samples or between ASM-positive and ASM-deficient cells are indicated by an asterisk* or a delta Δ, respectively.

3.3 Role of P-selectin in Acid Sphingomyelinase Activation

3.3.1 Deficiency of P-selectin prevents Acid Sphingomyelinase Activation

The adhesion receptor P-selectin is known to be involved in the interaction of platelets with tumor cells. When platelets derived from P-selectin-deficient mice and normal mice were co-incubated with B16F10 melanoma cells, the deficiency of P-selectin in platelets prevented activation of acid sphingomyelinase and subsequent release of ceramide, while these events rapidly occurred in platelets obtained from wild-type mice (Fig. 8).

Fig. 8:

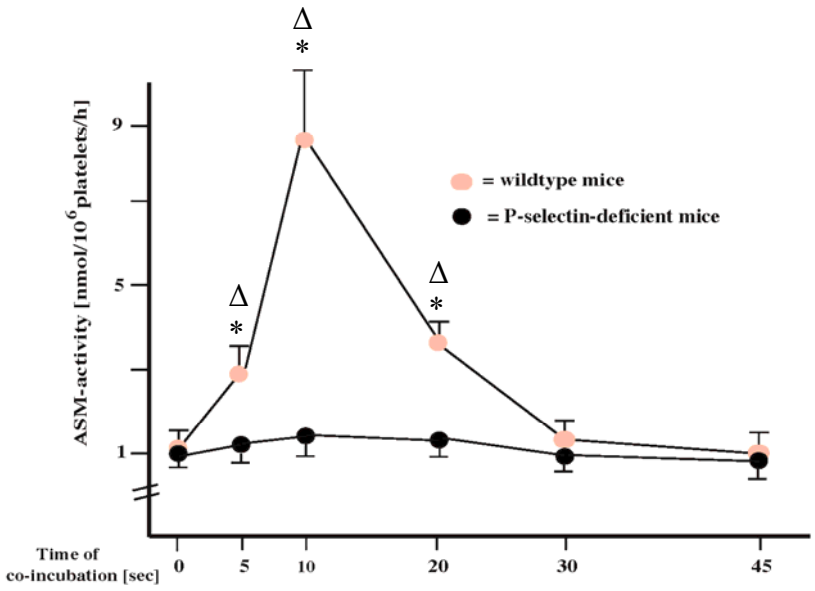


Fig. 8: P-selectin mediates activation of acid sphingomyelinase in platelets upon co-incubation with B16F10 melanoma cells

B16F10 melanoma cells rapidly stimulated acid sphingomyelinase (ASM) in P-selectin-positive platelets, while no activation of the enzyme was observed in P-selectin-deficient platelets upon co-incubation with tumor cells. Platelets from normal or P-selectin-deficient mice were purified, incubated with B16F10 tumor cells and the activity of acid sphingomyelinase was determined by consumption of [^{14}C]-sphingomyelin. The results are the mean ± SD of 4 independent studies.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated samples or between P-selectin-positive and P-selectin-deficient cells are indicated by an asterisk* or a delta Δ, respectively.

3.3.2 P-selectin-deficient platelets fail to release ceramide

Wild-type platelets and platelets obtained from P-selectin-deficient mice were co-incubated with B16F10 melanoma cells, and subsequently the amount of ceramide released was measured in a DAG kinase assay. While wild-type platelets released ceramide upon co- incubation, platelets that did not express P-selectin showed no release of ceramide (Fig. 9). In wild-type platelets, the amount of ceramide released peaked after about 15 seconds and declined back to baseline within 60 seconds, while in P-selectin-deficient platelets the amount of ceramide released maintained baseline level.

Fig. 9:

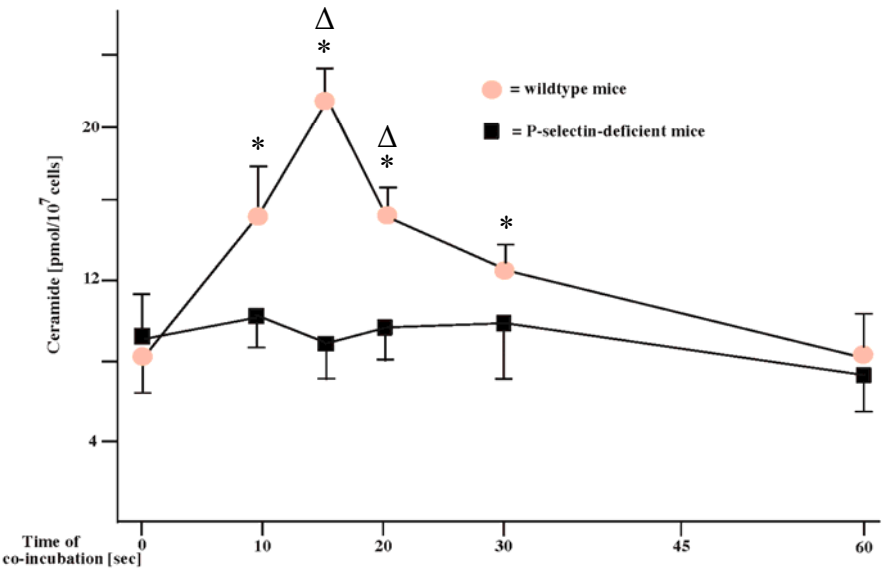


Fig. 9: P-selectin mediates release of ceramide in platelets upon co-incubation with B16F10 melanoma cells

Incubation of P-selectin-positive platelets with B16F10 tumor cells triggered the release of ceramide within 10 seconds, as determined by the DAG-kinase assay. P-selectin-negative platelets failed to release ceramide upon stimulation with B16F10 tumor cells. Ceramide concentrations in the tumor cells were subtracted from the total values. Shown is the mean ± SD of 3 independent experiments.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated samples or between P-selectin-positive and P-selectin-deficient cells are indicated by an asterisk* or a delta Δ, respectively.

3.3.3 Intracellular Molecule and Tyrosine-Phosphorylation

Failure of P-selectin-deficient platelets to activate acid sphingomyelinase after co-stimulation with B16F10 melanoma cells was not caused by a reduced interaction of platelets with tumor cells, because other signaling events elicited by tumor cells in platelets were not affected, as is shown by western blotting in Fig. 10 A-D. Experiments revealed a very similar induction of cellular tyrosine phosphorylation (Fig. 10 A) and phosphorylation of p44/42 mitogen-activated protein kinase (MAP-K, Fig. 10 B), c-Jun N-terminal kinase (JNK, Fig. 10 C) or phospholipase Cγ (Fig. 10 D) in P-selectin-positive and -negative platelets when co-stimulated with B16F10 melanoma cells. These enzymes are typical markers of cellular activation and represent major signaling pathways in many cells, leading to reorganization of the cytoskeleton, calcium increase or PKC activation, for example.

Fig. 10 A:

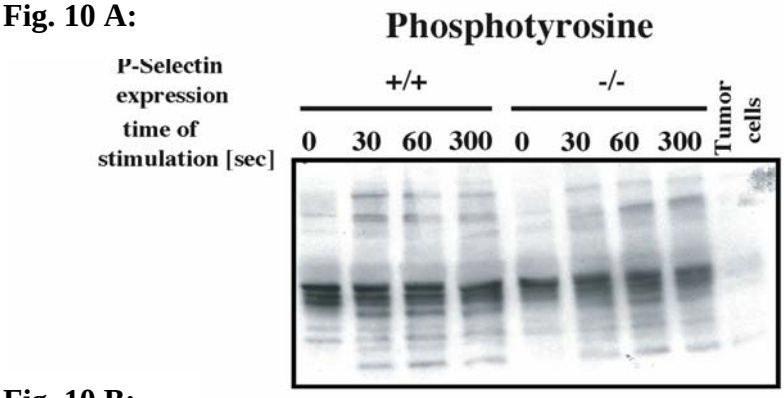


Fig. 10 B:

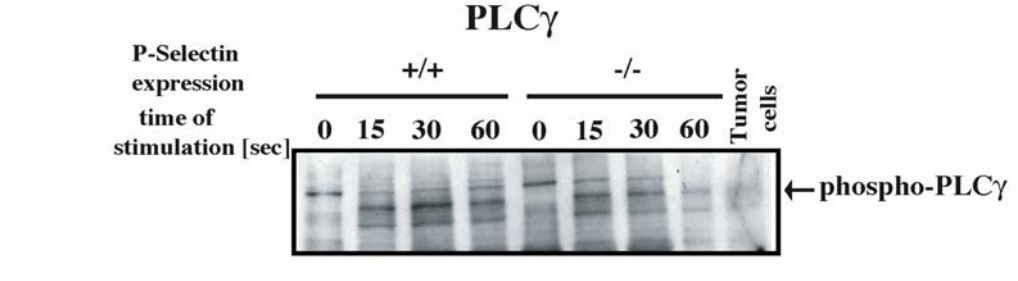


Fig. 10 C:

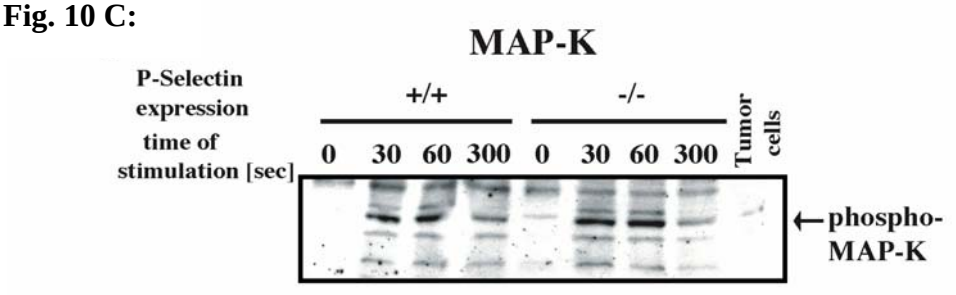


Fig. 10 D:

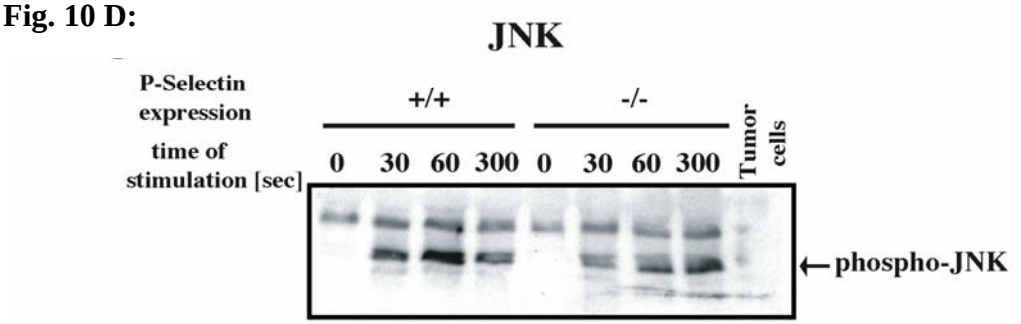


Fig 10 A-D: P- Selectin deficiency does not affect some signaling events in platelet-tumor cell interaction.

Incubation of platelets with B16F10 melanoma cells induced tyrosine phosphorylation of several intracellular molecules (**A**) and phosphorylation of PLCγ (**B**), MAP-K (**C**) and JNK (**D**) on residues that indicate activation of these molecules. However, deficiency of P-selectin in platelets did not significantly alter these events, indicating that B16F10 tumor cells are also able to activate platelets via pathways that are independent of P-selectin. Protein phosphorylation was determined by western blotting of whole cell lysates and detection with anti-phosphotyrosine, anti-phospho-PLCγ, anti-phospho-MAP-K or anti-phospho-JNK antibodies and development using the Tropix® chemiluminescence system.

3.3.4 P-selectin mediates B16F10 Tumor Cell Metastasis

To determine hematogenous metastasis of B16F10 melanoma cells, P-selectin-positive and -negative C57/Bl6 mice were intravenously injected with $1x10^5$ B16F10 tumor cells. After 21 days the mice were sacrificed, the lungs removed, and the number of metastases was determined. The results depicted in Fig. 11 reveal multiple pulmonary metastases in wild- type mice that were reduced by 94% in P-selectin-deficient mice.

Fig. 11:

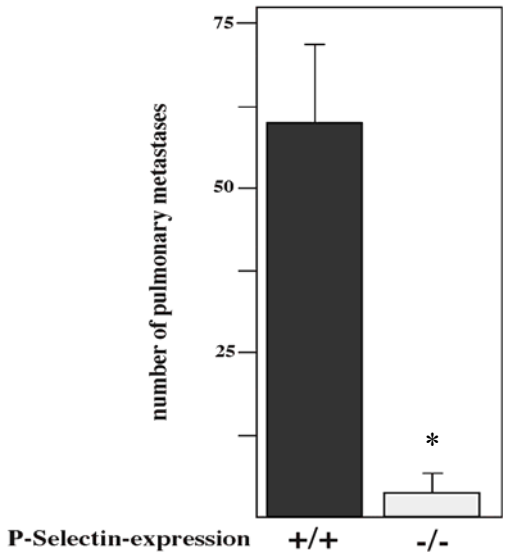


Fig. 11: P-selectin mediates B16F10 tumor cell metastasis

Metastasis of B16F10 tumor cells was prevented in P-selectin-deficient mice, while wild-type mice showed multiple tumor metastases in the lung. The figure demonstrates the quantitative analysis of B16F10 metastases in the lung and displays the mean ± SD of melanoma in the lungs 21 days after intravenous injection of 50,000 B16F10 melanoma cells into P-selectin-positive or -deficient syngenic C57/Bl6 mice. Represented are the mean ± SD from 11 wild-type and 10 P-selectin-deficient mice.

3.3.5 Co-incubation of platelets with B16F10 melanoma cells leads to P-selectin and GPIIbIIIα translocation independently of the acid sphingomyelinase

To determine the significance of the acid sphingomyelinase in the degranulation and translocation of P-selectin and the platelet-specific glycoprotein GPIIbIIIα in platelets, $1x10^6$ platelets of both wild-type and acid sphingomyelinase-deficient mice were co-incubated with $1x10^5$ B16F10 melanoma cells. For FACS analysis, the FITC- labeled antibody α-P-selectin and the PE-labeled antibody α-GPIIbIIIα were used. Results showed that degranulation occurred in wild-type platelets and P-selectin and GPIIbIIIα were translocated to the outer cellular membrane (Fig. 12, left panel). These events were not altered by deficiency of the acid sphingomyelinase in platelets (Fig. 12, right panel), indicating that the process of degranulation and translocation of the P-selectin molecule and the GPIIbIIIα molecule occurs independently of the presence of acid sphingomyelinase.

Fig. 12:

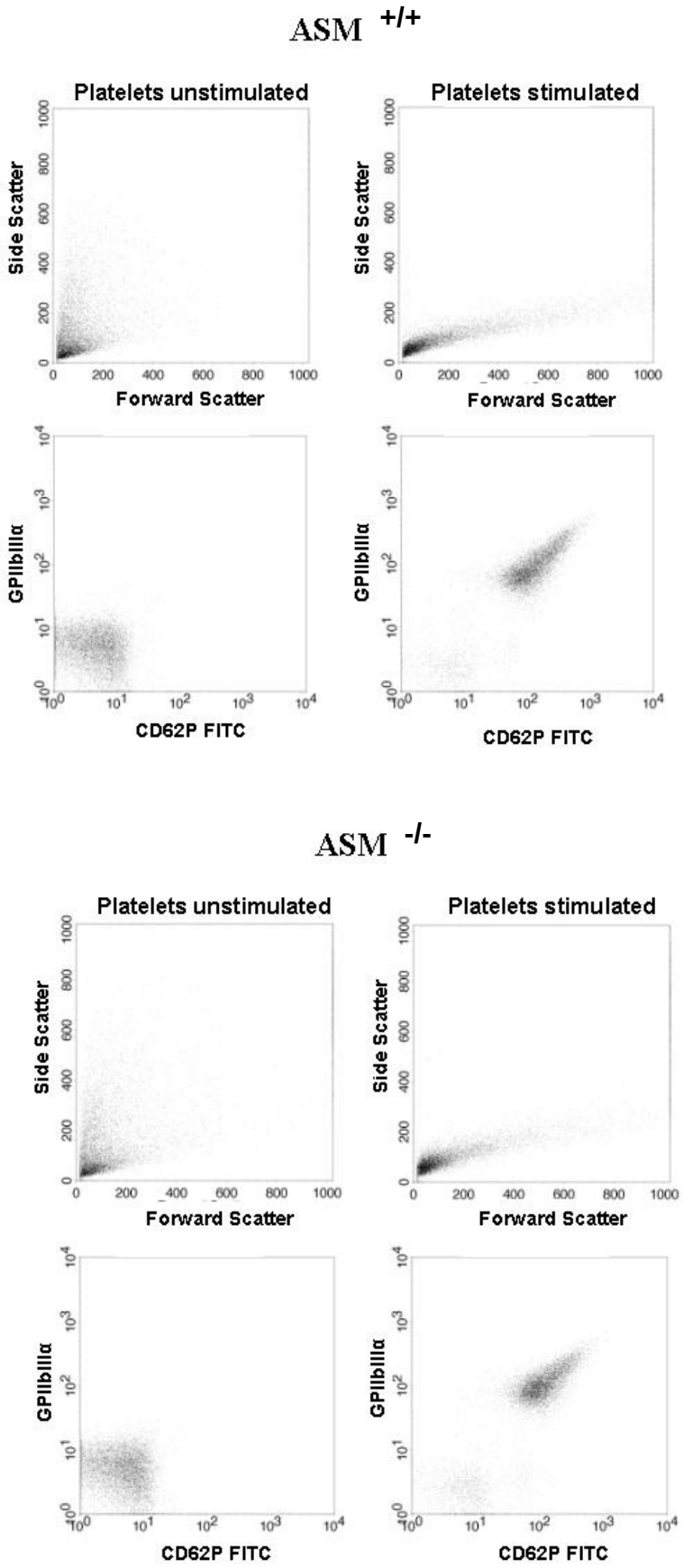

ASM +/+
Platelets unstimulated
Platelets stimulated
Side Scatter
Forward Scatter
GPIIbIIIα
CD62P FITC
ASM -/-
Platelets unstimulated
Platelets stimulated
Side Scatter
Forward Scatter
GPIIbIIIα
CD62P FITC

Fig. 12: Platelet degranulation and P-selectin/GPIIbIIIa expression after stimulation with B16F10 melanoma cells occurs in wild-type and ASM-deficient Platelets.

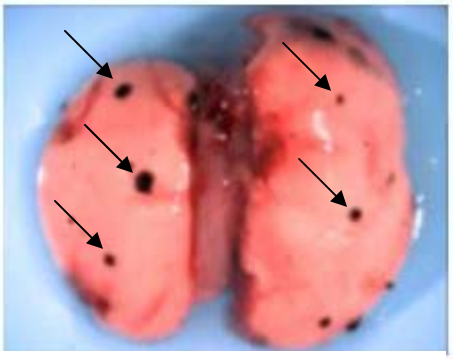
$1x10^6$ Platelets were obtained from either wild-type or acid sphingomyelinase-deficient C57/Bl6 mice and stimulated with $1x10^5$ B16F10 melanoma cells. The antibodies for P-selectin and GPIIbIIIα were added to the samples and FACS analysis was carried out. The left panel (indicating wild-type platelets used) shows degranulation and a decrease in size of the platelets and expression of P-selectin and GPIIbIIIα. Acid sphingomyelinase-deficient platelets (right panel) did not significantly differ from wild-type platelets, suggesting that degranulation and P-selectin and GPIIbIIIα expression occurs independently of the presence of the acid sphingomyelinase.

3.4 Fucoidan and Tumor Metastasis

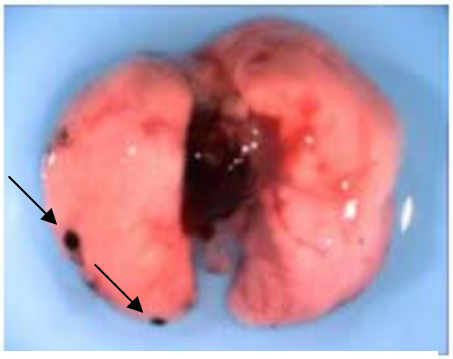
3.4.1 Fucoidan prevents B16F10 tumor metastasis in mice

Wild-type mice were injected with various amounts of fucoidan, and after one hour were injected with B16F10 melanoma cells. Upon sacrifice of the mice after two weeks and removal of the lungs, the number of metastases was determined. Results (Fig. 13 A+B) revealed that while the control mice (injection of HEPES-Saline instead of fucoidan) exhibited high numbers of metastases in the lung (mean=48 ± 18), there is a gradual decrease in the number of pulmonary metastases. When 0.08 mg/mouse fucoidan was administered, the mice exhibited a mean number of 23 ± 10 metastases in the lung. This number was further reduced by the administration of 0.4 mg/mouse fucoidan to a mean of 5 ± 3 tumor metastases, and when 2.0 mg/mouse fucoidan was injected, metastasis was prevented.

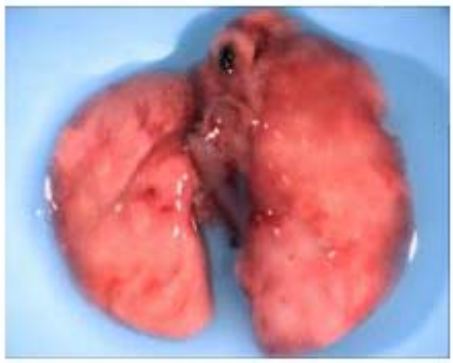
Fig. 13 A:



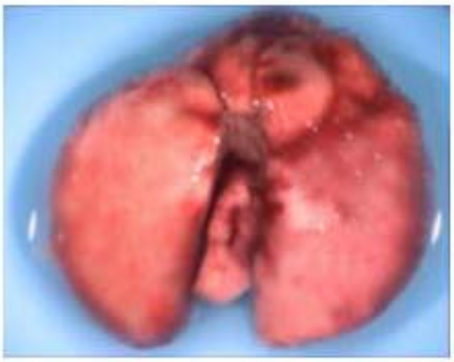
0 mg Fucoidan/Mouse



0.08 mg Fucoidan/Mouse



0.4 mg Fucoidan/Mouse



2.0 mg Fucoidan/Mouse

Fig. 13 B:

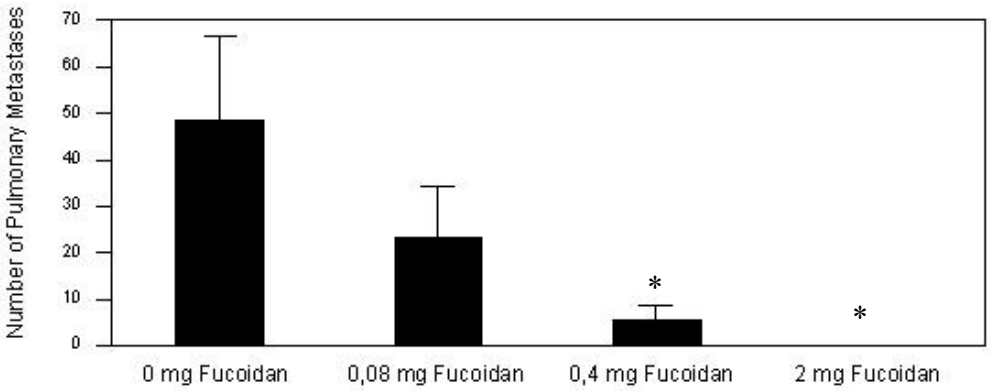


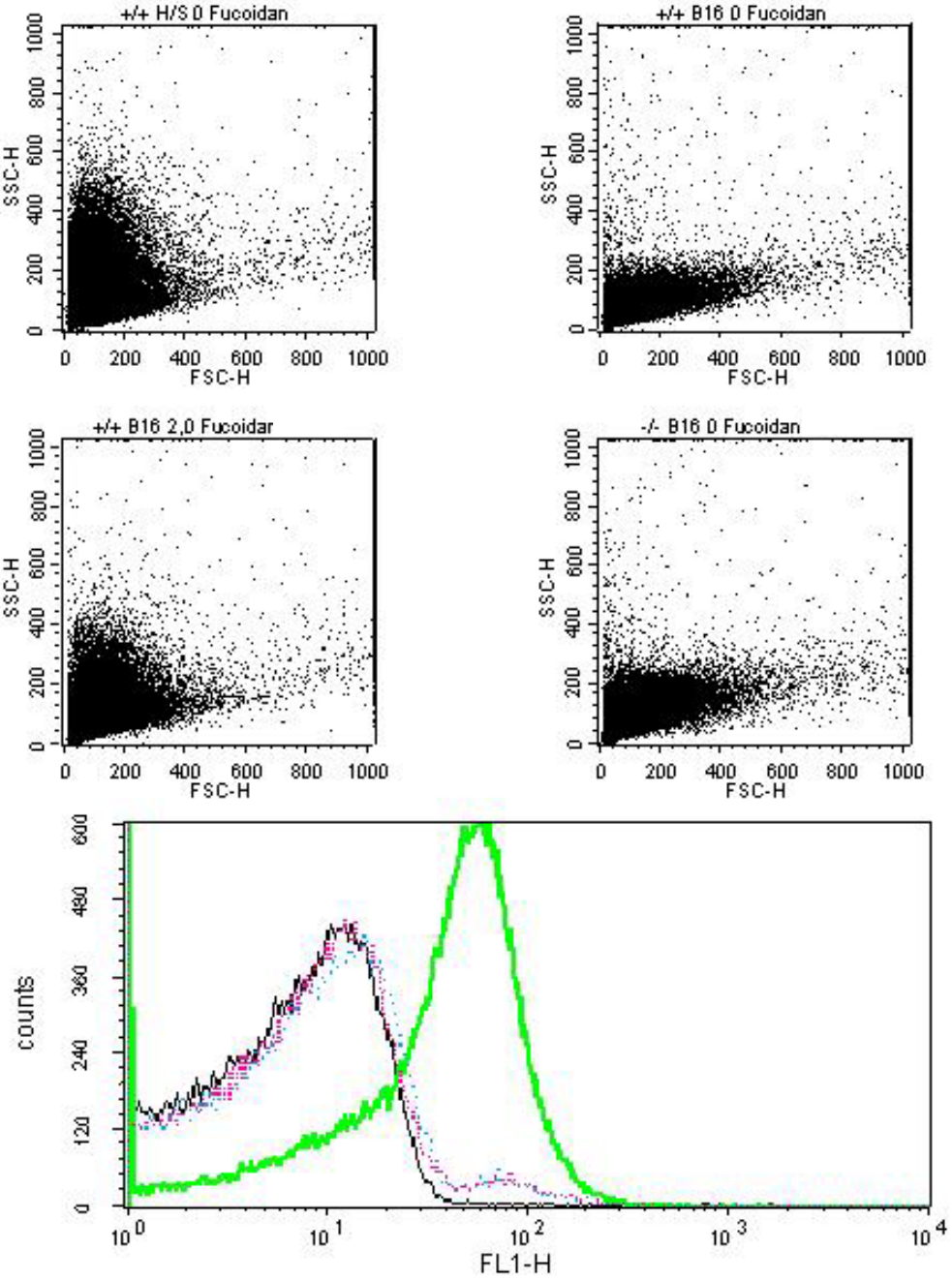
Fig. 13 A+B : Injection of fucoidan prevents B16F10 melanoma cell metastasis.

Intravenous injection of 2 mg/mouse of fucoidan 2 hours prior to intravenous injection of 1 x 10^5 B16F10 melanoma cells abolished tumor metastasis to the lung of C57/Bl6 mice (n=3 per condition). Mice were sacrificed by cervical dislocation 21 days after melanoma cell/fucoidan treatment, the lungs were removed and the visible metastases were counted. Panel **A** shows the results of a typical experiment (metastases at arrowheads). Panel **B** indicates the mean ± SD of three independent experiments.
Significant differences ($p \leq 0.05$, *t*-test) between fucoidan-treated and -untreated samples are indicated by an asterisk*.

3.4.2 FACS analysis of tumor cell-platelet interaction after treatment with fucoidan

When wild-type platelets or platelets from acid sphingomyelinase-deficient mice were co-incubated weith B16F10 melanoma cells in the presence of various amounts of fucoidan, FACS analysis revealed that when stained for P-selectin with P-selectin antibody, 2 mg/ml fucoidan was sufficient to block the interaction of platelets with B16F10 tumor cells (Fig. 14). While wild-type platelets co-incubated with tumor cells showed that platelets rapidly degranulated to translocate P-selectin to the cellular surface, this degranulation was abolished by the treatment of fucoidan and the platelets remained unactivated.

Fig. 14:



Black: P-selectin +/+ H/S 0 Fucoidan
Green: P-selectin +/+ B16 0 Fucoidan
Pink: P-selectin +/+ B16 2,0 Fucoidan
Blue: P-selectin -/- B16 0 Fucoidan

Fig. 14: Fucoidan prevents activation through B16F10 co-incubation of thrombocytes.

While co-incubation of wild-type thrombocytes with HEPES-saline caused no effect in thrombocytes (top panel, left), co-incubation of 1 $x10^6$ wild-type thrombocytes with 1 $x10^5$ B16F10 melanoma cells rapidly activates thrombocytes to degranulate and expose P-selectin molecule to the outer surface of the cell (top panel, right), as detected by α-P-selectin antibody. When 2.0 μg/mouse fucoidan was added to the thrombocytes prior to B16F10 melanoma cell addition, no activation or degranulation of the thrombocytes was detected by α-P-selectin antibody (bottom panel, left). P-selectin-deficient thrombocytes, when co-incubated with B16F10 melanoma cells in the absence of fucoidan, were still activated and degranulated (bottom panel, right), indicating the lack of P-selectin itself does not prevent activation of thrombocytes by B16F10 melanoma cells. These findings are represented in the bottom graphical analysis.

3.5 Transplantation of Wild-Type Bone Marrow reconstitutes B16F10 Tumor Metastasis in P-selectin-Deficient Mice

To discriminate whether P-selectin in platelets or endothelial cells mediates B166F10 metastasis, P-selectin wild-type and P-selectin-deficient mice were irradiated and transplanted with bone marrow from P-selectin wild-type or P-selectin-deficient mice, sacrificed after 21 days, the lungs were removed, and visible metastases were counted. Deletion of P-selectin in thrombocytes, i.e. wild-type mice transplanted with P-selectin-deficient bone marrow, abolished tumor metastasis of intravenously injected tumor cells, while deficiency of P-selectin in endothelial cells, i.e. P-selectin-deficient mice transplanted with wild-type bone marrow, did not significantly influence tumor metastasis (Fig. 15). Injection of P-selectin wild-type bone marrow in P-selectin$^{-/-}$ mice reconstituted tumor metastasis. These results indicate that thrombocytic P-selectin plays a significant role in tumor metastasis of B16F10 melanoma cells in the lungs of mice, while endothelial P-selectin expression is of less importance.

Fig. 15:

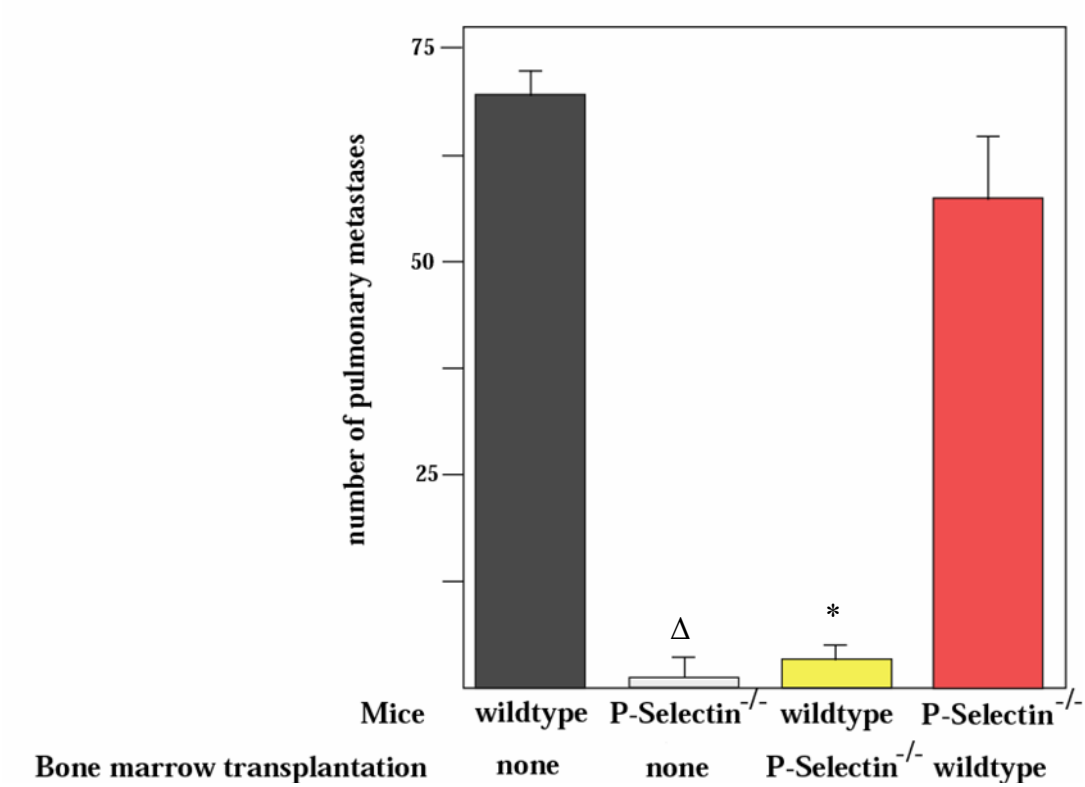


Fig. 15: Transplantation of wild-type bone marrow in P-selectin-deficient mice reconstitutes B16F10 melanoma cell metastasis.

Wild-type or P-selectin-deficient mice were irradiated and transplanted with P-selectin-deficient or wild-type bone marrow. This resulted in mice having P-selectin-deficient thrombocytes and normal endothelial cells, or wild-type thrombocytes and P-selectin-deficient enothelial cells. These mice, as well as non-transplanted, P-selectin-deficient, and wild-type mice, were injected intravenously with $5x10^4$ B16F10 melanoma cells, and the number of visible pulmonary metastases was determined after 21 days. The graph shows the mean ± SD of pulmonary metastases of three mice per experiment.
Significant differences ($p \leq 0.05$, *t*-test) between wildtype mice transplanted or not with P-selectin-deficient bone marrow are indicated by an asterisk*, while differences between P-selectin-deficient mice transplanted or not with wildtype bone marrow are indicated by a delta Δ.

4. Discussion

4.1 Discussion of the Methods

4.1.1 DAG-kinase assay for ceramide measurement

The importance of the development of rapid and quantitative assays for the measurement of ceramide levels is revealed in the crucial role of ceramide in several cellular processes and stress responses in particular. Methods that have been developed for the quantification of ceramide include normal phase HPLC analysis after derivatization with a fluorescent tag (Iwaromi *et al.* 1979; Previati *et al.* 1996; Couch *et al.* 1997; Yano *et al.* 1998) and cellular labeling with radioactive precursor (Tepper *et al.*, 2000). The liberated and derivatized sphingoid bases can be used after ceramide is being hydrolyzed via HPLC (Smith *et al.*, 1995) and fatty acids by means of gas chromatography/mass spectrometry (Samuelsson *et al.*, 1970).

The diacylglyceride (DAG or DG) assay has emerged to become the most common method for ceramide quantification with the advantages of the assay being the measurement of total mass levels of ceramide, usage of crude lipid extracts, and the ability that a large number of samples can be processed in a rapid manner. Hokin and Hokin originally reported the DAG activity (Hokin and Hokin, 1959), showing the linear relationship between the amount of diglyceride added to an *in vitro* assay and the amount of phosphatidic acid formed (Kennerly *et al.*, 1979). Structurally, ceramides share similarities with diglycerides, and it was shown that bacterial DAG kinase can utilize ceramide as a substrate nearly five times greater than that for diglycerides (Schneider and Kennedy, 1973). Modifications of the assay demonstrated that DAG kinase could also be used for quantitative conversion of ceramide-1-phosphate.

Ceramide is a nonpolar molecule; therefore, there is a need that it be extracted from cells after cell lysis in organic solvents like chloroform and methanol. Acidifying the lysate by the addition of hydrochloric acid aids in the extraction of shorter acyl chain ceramide-1-

phosphates or hydroxylated ceramides. In the DAG kinase reaction, it is cucial that the substrate be presented to the enzyme in a soluble form for the most effective conversion to product. To this end, mixed micelles that contain a non-ionic detergent like n-octyl-β-D-glycopyranoside and a phospholipid like cardiolipin are being used. The level of ceramide conversion to ceramide-1-phosphate is of particular importance in this assay since the extraction must complete with a total conversion of DAG and ceramide; otherwise, the results will be influenced by the effects of efficiency of the reaction and to possible exclusion between DAG and ceramide substrates. The phosphorylated substrates DAG and ceramide are easily separated via thin layer chromatography.

4.1.2 Acid sphingomyelinase activity determination

The determination of acid sphingomyelinase enzyme activity in cell lysates requires sphingomyelin labeling by radioactivity or fluorescence. In this study, the enzymatic activity was measured by the degradation of radioactive [^{14}C]-sphingomyelin to ceramide and phosphorylcholine. The radioactive label is attached to the choline group and is soluble in water, the radioactivity that is released can be separated from the substrate because the latter partitions to the organic phase after extraction. By performing the assay in this study at a pH of 5.0, acid sphingomyelinase activity was discriminated from neutral and basic sphingomyelinases, which also utilize the substrate sphingomyelin. Because of the difficulty in bringing together the enzyme and substrate molecules in the presence of cell homogenates, a suitable detergent, such as Triton X-100 was used. It should be noted that, apart from emulsifying the substrate, the detergents bind and modify the enzyme activity, thus detergent concentration and initial detergent:substrate ratio was kept constant for reproducibility of assays. Finally, it has been suggested that the ASM may be located in detergent-resistant/insoluble fractions; thus, for determination of ASM activity in whole-cell lysates, no centrifugation step was performed after lysis and sonication of the cells to prevent pelleting and loss of the ASM.

4.1.3 Transgenic Animals

4.1.3.1 Acid sphingomyelinase-deficient mice

To determine the role and importance of acid sphingomyelinase in melanoma cell metastasis, thrombocytes obtained from acid sphingomyelinase-deficient mice were obtained. The acid sphinomyelinase knock-out mice were originally generated in the laboratory of Dr. Edward Schuchman (Mount Sinai School of Medicine, New York, NY, USA). These mice have been a valuable tool in the investigation of the role of the acid sphingomyelinase enzyme role in several different cellular processes. It was shown that acid sphingomyelinase-deficient mice generated by gene targeting abundantly store sphingomyelin in the reticuloendothelial system of liver, spleen, bone marrow, and in brain (Nix *et al.*, 2000). Liver cells of acid sphingomyelinase-deficient mice accumulate sphingomyelin and glycosphingolipids in purified lipid bilayers of microsomes, Golgi, and the plasma membrane, but cholesterol is depleted in the plasma membrane. Studies from Lozano and Kolesnick pointed out that mice displayed only a minimal increase in sphingomyelin content up to an age of 12-16 weeks, and unchanged levels of caveolin-1, normal MAP-kinase signaling, normal tyrosine phosphorylation patterns, absence of lymphopenia, normal T-cells and no decrease in FLIP levels (Lozano *et al.*, 2001) suggesting that these mice are a suitable model for studying the function of the ASM.

Hence, all experiments involving these mice were carried out with animals younger than 12 weeks of age to permit analysis of acid sphingomyelinase function of the effects of sphingomyelin accumulation.

4.1.3.2 P-selectin-deficient mice

In this study the P-selectin-deficient mice were obtained from Jackson laboratories. P-selectin is suggested to act by controlling the adhesion of tumor cells to vascular endothelial cells. Since tumor cell-induced activation of platelets is considered a crucial step in tumor metastasis, it is assumed that a mechanism for tumor metastasis might be the

stimulation of expression of platelet P-selectin (Krause and Turner, 1999), leading to the formation of tumor cell:platelet aggregates that could form emboli in small vessels or adhere to endothelial cells and initiate metastatic deposition (Krause and Turner, 1999). It was, however, determinded by Hakomori *et al.* (Hakomori, 1996) that tumor cells that were able to activate platelets were not capable of binding to P-selectin, yet Stone and Wagner (1993) could show the P-selectin-dependent binding of platelets to two small lung tumor and two neuroblastoma cell lines. Interestingly, it was shown (Kates and Geng, 1998) that activated platelets increased adhesion of melanoma cells to endothelial cells under flow conditions which was mainly due to platelet GPIIb/IIIα. P-selectin-deficient mice that were subcutaneously implanted with tumor cells showed significantly slower tumor growth (Kim *et al.*, 1998), and fewer lung metastases were observed with intravenously injected tumor cells. These studies also showed that platelets from the P-selectin-deficient mice failed to adhere to tumor cell surface mucins and the lungs exhibited significantly fewer platelet clumps. Interestingly, Nooijen *et al.* (1998) observed that P-selectin is absent from the microvasculature in advanced primary melanoma and melanoma metastasis as compared to benign melanoma and normal skin, suggesting that advanced melanoma may evade inflammatory regression by decreased endothelial P-selectin expression.

4.2 Discussion of the Results

4.2.1 Acid sphingomyelinase deficiency attenuates tumor metastasis

Hematogenous metastasis occurs when tumor cells enter the blood stream, interact with host blood cells, avoid host defense system, arrest on the endothelial cells in the blood stream, extravasate and colonize a distant tissue. Evidence indicates that hematogenously disseminating tumor cells interact with platelets and leukocytes, forming microemboli that facilitate their arrest in the vasculature (Karpatkin and Pearlstein, 1981; Gasic, 1984; Fidler, 1990; Honn *et al.*, 1992). A prominent feature of tumor cells is an altered cell surface glycosylation (Fukuda, 1996; Hakomori, 1996; Kim *et al.*, 1996; Kannagi, 1997; Kim and Varki, 1997); in particular, sialylated fucosylated glycan expression like sialyl-

Lewis-x and sialyl-Lewis-a correlate with poor prognosis due to tumor progression and high rate of tumor metastasis. Certain sialyl Lewis- x/a glycans found on leukocytes and endothelium are recognized by vascular cell adhesion molecules such as selectins (Fukuda, 1996, Takada *et al.* 1993), mediating tumor cell interaction with platelets, leukocytes and endothelial cells (Varki and Varki, 2002; Mannori *et al.*, 1995; Kim *et al.*, 1999; Izumi *et al.*, 1995). Platelets adhere to some human carcinoma cells mostly via P-selectin and inhibition of this interaction leads to attenuation of metastasis *in vivo* (Kim *et al.*, 1998; Borsig *et al.*, 2001). Additionally, platelet adhesion to tumor cells physically interferes with access of leukocytes to tumor cells, suggesting a possible shielding effect (Nieswandt *et al.*, 1999; Borsig *et al.*, 2001).

In this present study it was shown that acid sphingomyelinase-deficient C57/Bl6 mice showed a significantly reduced number of metastases in the lung after 21 days of injection of B16F10 melanoma cells as compared to wild type C57/Bl6 mice (Figure 1 A+B), therefore suggesting a pivotal role of the acid sphingomyelinase enzyme in the process of tumor metastasis of melanoma cells. A possible role of acid sphingomyelinase in tumor metastasis has not previously been shown, and signaling events elicited by tumor cells in platelets are not characterized. It has been previously shown that the acid sphingomyelinase is very rapidly activated by a number of stress and pro-apoptotic stimuli to release ceramide that forms large ceramide-enriched membrane platforms and therefore reorganize the cellular membrane to facilitate signaling into the cell. Several pro-apoptotic receptor molecules like CD95 (Cifone *et al.*, 1994; Gulbins *et al.*, 1995; Brenner *et al.*, 1998), TNF-R (Schutze *et al.*, 1992) or IL-1 (Mathias *et al.*, 1993), amongst others, have been demonstrated to activate the acid sphingomyelinase and to result in a release of ceramide. Genetic experiments on acid sphingomyelinase-deficient mice have shown the important role of acid sphingomyelinase for induction of apoptosis by these stimuli (Gulbins, 2002). Activation of the acid sphingomyelinase in platelets by tumor cells is mediated by P-selectin as shown by the failure of P-selectin-deficient platelets to stimulate the acid sphingomyelinase and to release ceramide. This demonstrates a novel function of ceramide in the activation of platelets, and a novel mechanism of tumor metastasis.

In order to identify signal transduction events that mediate the activation of thrombocytes by tumor cells and their metastatic capabilities, the activity of the acid

sphingomyelinase was measured after incubation with purified B16F10 tumor cells. Co-incubation of wild type platelets with B16F10 tumor cells over a time course up to 1 minute showed that within the first 5 seconds the acid sphingomyelinase in the thrombocytes was stimulated, and this activity peaked after 10 seconds of the begin of the interaction between tumor cells and thrombocytes. 60 seconds after tumor cell:platelet interaction the activity of acid sphingomyelinase returned to base line levels. Co-incubation of thrombocytes purified from acid sphingomyelinase-deficient mice with B16F10 melanoma cells did not reveal any additional activity of the acid sphingomyelinase compared to baseline activity, indicating that by incubation of thrombocytes with B16F10 melanoma cells the raised activity of the acid sphingomyelinase enzyme is due to the acid sphingomyelinase in the thrombocytes and not the tumor cells.

4.2.2 Acid sphingomyelinase activity and ceramide production

The activation of the acid sphingomyelinase correlated with the production of ceramide after stimulation of the thrombocytes with B16F10 melanoma cells. The formation of ceramide was determined by the DAG kinase assay after stimulation of thrombocytes with tumor cells. Compared to wild-type thrombocytes, acid sphingomyelinase-deficient thrombocytes failed to release ceramide. This shows that the release of ceramide after co-incubation of tumor cells with thrombocytes is dependent on the expression of acid sphingomyelinase in thrombocytes.

It has been shown that P-selectin deficiency abrogates tumor metastasis in mice, indicating that P-selectin plays a key role in tumor metastasis. It is a novel finding that a link has been established between P-selectin-deficiency and the release of ceramide. In this study P-selectin-deficient thrombocytes were co-incubated with B16F10 melanoma cells over a time course. As wild-type platelets from C57/Bl6 mice showed an activation of the acid sphingomyelinase where the acid shingomyelinase activity peaked after 10 seconds of co-incubation with B16F10 melanoma cells and declined thereafter, reaching baseline levels again after app. 30 seconds, thereby indicating a rapid activation, P-selectin-deficient thrombocytes showed an acid sphingomyelinase activity at baseline

levels (Figure 4). The activation of acid sphingomyelinase in wild-type thrombocytes correlated with the release of ceramide, peaking after app. 15 seconds and declining thereafter, while co-incubation of ASM-deficient thrombocytes with melanoma cells failed to release ceramide (Figure 5). It can be concluded that since there is no acid sphingomyelinase activity, the deficiency of activity of the enzyme is therefore the cause of the failure of ceramide release from sphingomyelin.

4.2.3 B16F10 tumor cells also activate platelets via pathways independent of P-selectin

To exlude the possibility that the failure of activation of the acid sphingomyelinase and the subsequent release of ceramide in P-selectin-deficient thrombocytes is due to inhibition of thrombocyte:tumor cell contact and interaction, it was determined whether P-selectin-deficient thrombocytes and wild-type thrombocytes exhibited cellular tyrosine phosphorylation as well as activity of PLCγ, JNK and MAPK after co-incubation of the thrombocytes with tumor cells. The signaling events were stimulated in wild-type as well as P-selectin-deficient thrombocytes, indicating that the tumor cells interacted with wild-type as well as P-selectin-deficient thrombocytes and hitherto activate either set albeit one being P-selectin-deficient. In fact, Figure 10 indicates that there is no difference in tyrosine phosphorylation, activation of PLCγ, MAPK, and JNK, in wild-type thrombocytes compared to P-selectin-deficient thrombocytes upon co-incubation thereof with B16F10 melanoma cells, indicating that B16F10 tumor cells are also able to activate platelets via pathways that are independent of P-selectin. Generally, these enzymes are typical markers of cellular activation and represent major signaling pathways in many cells. MAP kinase is a threonine/serine specific protein kinase that responds to extracellular stimuli (mitogens) and regulates various cellular activities such as gene expression, mitosis, differentiation and cell survival or apoptosis (Springer, 1995). The extracellular stimuli activate MAP kinase by a signaling cascade, being phosphorylated on its serine and threonine residues. MAP kinases are involved in the action of most non-nuclear oncogenes and are responsible for cell response to growth factors. JNK are involved in a signaling cascade, being activated by stress factors like UV-radiation, interleukins, or chemokines, amongst others. The targets of JNK are proteins within the

cellular nucleus, esp. the phosphorylation of c-JUN that regulates gene expression as apoprotein-1, proteins of the cytoskeleton, as well as members of the BH3 family, where the latter inactivates the anti-apoptotic Bcl-2 by phosphorylation, and pro-apoptotic proteins like Bax are activated. PLCγ is part of a G-protein-coupled receptor signal transduction pathway and is activated by transmembrane receptors with intrinsic or associated tyrosine kinase activity. PLCγ hydrolyzes phosphatidylinositol into two second messengers, inositol triphosphate and diacylglycerol (DAG). These products then modulate the activity of downstream proteins during cellular signaling like calcium channels in the endoplasmic reticulum of several different cell types and protein kinase C. PLCγ requires calcium for catalytic activity and is activated by receptor tyrosine kinase.

4.2.4 Platelets degranularize independently of P-selectin

When activated, thrombocytes degranularize and expose P-selectin, stored in the intracellular alpha granules, on the cellular surface, as well as GPIIbIIIα, a platelet-specific receptor. When wild-type thrombocytes are activated in co-incubation with B16F10 melanoma cells, they rapidly degranularize and reduce in size (Figure 12). Degranularization exposes GPIIbIIIα and P-selectin on the extracellular surface, as determined by specific antibodies. Degranularization of thrombocytes and their size reduction also occurs in thrombocytes deficient for acid sphingomyelinase, indicating that the translocation of P-selectin and GPIIbIIIα occurs independently of the presence of acid sphingomyelinase.

Since P-selectin is expressed both on endothelial cells as well as thrombocytes, it was determined by transplantation experiments that wild-type bone marrow reconstituted the ability in P-selectin-deficient mice to form metastases. Since deletion of P-selectin in thrombocytes abolished tumor metastasis of intravenously injected tumor cells in wild-type mice, and deficiency of endothelial cell P-selectin (P-selectin-deficient mice) reconstituted tumor metastasis, it indicates that thrombocytic P-selectin plays a significant role in tumor metastasis of B16F10 melanoma cells in the lung, while endothelial P-selectin is of less importance, at least under these circumstances.

4.2.5 Fucoidan binds and blocks P-selectin

It stands to reason that blocking P-selectin protein on thrombocytes could be of great medical importance in the course of cancer treatment. P-selectin binds sulfated molecules such as heparin and sulfatides (Varki, 1994; Crocker and Feizi, 1996). All selectins bind with higher affinity to only a few glycoproteins on leukocytes or endothelial cells. These glycoproteins must be sialylated and fucosylated to interact with selectins. For P- and L-selectins, they must be sulfated (Imai *et al.*, 1993; Hemmerich *et al.*, 1994; Wilkins *et al.*, 1995; Pouyani and Seed, 1995; Sako *et al.*, 1995; Li *et al.*, 1996), and binding requires sialylated and fucosylated O-glycans. Fucoidan is a sulfated polysaccharide present in brown seaweed, and it has been reported (Durig *et al.*, 1997; Patankar *et al.*, 1993) to be a heparin-like molecule. Fucoidan has anti-coagulatory properties (Durig *et al.*, 1997; Trento *et al.*, 2001) and inhibitory effects on tube formation of human endothelial cells (Soeda *et al.*, 2000) in addition to anticancer effects through modulation of host immune systems (Riou *et al.*, 1996; Itoh *et al.*, 1993; Zhuang *et al.*, 1995; Koyanagi *et al.*, 2003). In this study, fucoidan was injected into mice shortly before injection of B16F10 melanoma cells. The results show that fucoidan inhibited tumor metastasis, most likely by interfering with thrombocytic P-selectin. As P-selectin is blocked by action of this sulfated polysaccharide, thrombocytes no longer are able to bind tumor cells by P-selectin, and the tumor cell:thrombocyte complex can no longer bind endothelial P-selectin and initiate rolling under shear stress and eventually halt on the inner lining of the vasculature. Therefore, extravasation does not take place with the effect of a decreased amount of metastases in the lungs of mice.

4.3 Ceramide, its Metabolites and Selectins

4.3.1 Ceramide triggers the release of Weibel-Palade P-Selectin

Current knowledge about the function of the acid sphingomyelinase, ceramide and its metabolites sphingosine and sphingosine-1-phosphate in platelets is limited. It has previosly been demonstrated that thrombin induces a release of the acid sphingomyelinase from platelets, but the functional consequences of acid sphingomyelinase secretion are not yet defined. Further studies suggested that sphingosine and sphingosine-1-phosphate support the aggregation of platelets, while the role of ceramide in platelet aggregation remains undefined. Endogenous ceramide triggers the release of P-selectin by Weibel-Palade body exocytosis (Bhatia *et al.*, 2004).

Ceramide and its metabolites mediate vascular inflammation by increasing endothelial cell expression of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule, thereby inducing leukocyte and monocyte adhesion to the vascular wall (Kim *et al.*, 2001; Xia *et al.*, 1998; Modur *et al*., 1996). These activated leukocytes and macrophages secrete cytokines that further induce inflammation and cytokine production. Ceramide is involved in generation of reactive oxygen species which can lead to inflammation and tissue injury (Bhunia et al, 1997). By triggering Weibel-Palade body exocytosis, ceramide increases endothelial P-selectin expression, which can induce leukocyte adhesion and aggregation to the vascular wall. Weibel-Palade bodies are located in endothelial cells only, therefore P-selectin expression is limited to that cell type. There is no evidence of yet that ceramide triggers the release of P-selectin in thrombocytes consistent with our findings.

4.3.2 Ceramide and reactive oxygen species

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the capacity of the cell to detoxify these potentially injurious oxidants using endogenous antioxidant defense systems. Conditions associated with oxidative stress include

ischemia/reperfusion, hypercholesterolemia, diabetes, and hypertension. The adhesion of circulating blood cells (leukocytes, platelets) to vascular endothelium is a key element of the pro-inflammatory and prothrombogenic phenotype assumed by the vasculature in these and other disease states that are associated with an oxidative stress. There is a growing body of evidence that links the blood cell endothelial cell interactions in these conditions to the enhanced production of ROS. Potential enzymatic sources of ROS within the microcirculation include xanthine oxidase, NAD(P)H oxidase, and nitric oxide synthase. ROS can promote a pro-inflammatory/prothrombogenic phenotype within the microvasculature by a variety of mechanisms, including the inactivation of nitric oxide, the activation of redox-sensitive transcription factors (e.g., nuclear factor-kappa B) that govern the expression of endothelial cell adhesion molecules (e.g., P-selectin), and the activation of enzymes (e.g., phospholipase A(2)) that produce leukocyte-stimulating inflammatory mediators (e.g., platelet-activating factor). The extensively documented ability of different oxidant-ablating interventions to attenuate blood cell:endothelial cell interactions underscores the importance of ROS in mediating the dysfunctional microvascular responses to oxidative stress.

4.4 Clinical Relevance of P-selectin-Mediated Acid Sphingomyelinase Activation in Tumor Metastasis

This study shows that metastasis of B16F10 melanoma cells requires an acid sphingomyelinase-triggered activation of platelets. Mice genetically deficient for acid sphingomyelinase are almost completely protected from tumor metastasis, indicating that pathophysiological significance of platelet acid sphingomyelinase for tumor metastasis *in vivo.* P-selectin mediates the activation of the acid sphingomyelinase as is shown by failure of P-selectin-deficient thrombocytes to stimulate the acid sphingomyelinase, release ceramide and develop lung metastases *in vivo* once challenged with melanoma cells. This study also indicates a novel function of ceramide in that it activates platelets by P-selectin. In melanoma cells, the course of metastasis is initiated by the binding of tumor cells by P-selectin ligands present on the surface of the tumor cells to platelets. This binding results in an intracellular activation of the platelets, indicating that the function of P-selectin is not merely adhesion but also serves as a receptor molecule that is

able to transmit signals into platelets. Thus far, it is unknown how P-selectin couples to acid sphingomyelinase and thereby mediates the release of ceramide. Activation of the acid sphingomyelinase appears to be independent of tyrosine kinases because activation of the acid sphingomyelinase is abrogated by deficiency of P-selectin while tumor cells elicit cellular tyrosine phosphorylation independent of P-selectin in platelets.

It has been demonstrated (Aigner *et al*., 1998) that thrombin induced a release of acid sphingomyelinase from platelets, although the functional consequences of its secretion remained undefined. Furthermore, sphingosine and sphingosine-1-phosphate support aggregation of platelets, while the role of ceramide in platelets has not been well defined. In cellular stimulation, acid sphingomyelinase has the general function to form ceramide-enriched membrane platforms that function to reorganize and cluster receptor molecules. Therefore, it can be concluded that acid sphingomyelinase and ceramide are not necessarily a part of the specific signaling cascade initiated by the activated receptor, but instead facilitate receptor signaling through ceramide-enriched membrane platforms by providing cellular conditions that allow receptors to transmit a specific signal. In the case of P-selectin, ceramide-enriched membrane platforms in platelets may re-organize and cluster the P-selectin molecules and/or receptors in the cellular membrane, resulting in a higher density of receptor molecules within a defined space in the platelet cellular membrane. This may facilitate and enhance contact between platelets, exposing P-selectin ligands and/or other receptor ligands, and endothelial cells, leading to a thrombus formation and finally the extravasation of the tumor cells. The increased receptor density in ceramide-enriched membrane platforms might be imperative to permit contact between platelets and endothelial cells even under shear stress as is present in the capillaries. It is not ruled out, however, that endogenous ceramide or its metabolites sphingosine or sphingosine-1-phosphate directly induce platelet aggregation by unknown intermediates, thereby forming the thrombus. Also, ceramide might change the conformation of adhesion receptors and increase the affinity to their ligands, resulting then in a thrombus formation. There is no mutual exclusion between the models, and it is possible that a combination of mechanisms mediates platelets and tumor cell adhesion to endothelial cells.

Clinically, in this study a link has been established that connects P-selectin-activated acid sphingomyelinase with tumor metastasis. The molecular mechanisms of tumor metastasis are currently not well understood, yet especially tumor metastasis is of great importance in the course of human cancer. The present studies identified the acid sphingomyelinase as a novel target molecule for the inhibition of tumor metastasis. Pharmaceutical agents that act on acid sphingomyelinase, i.e. acid sphingomyelinase inhibitors, could be utilized during surgical procedures with tumors or patients with tumors who exhibit high risk of tumor metastasis.

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Summary

The present manuscript demonstrates that B16F10 melanoma cells activate the enzyme acid sphingomyelinase in thrombocytes via the surface molecule P-selectin, by which ceramide is released. Metastasis of tumor cells in the lung is decreased by up to 95% by genetic deficiency of P-selectin molecule or deficiency of acid sphingomyelinase. After activation of wild type thrombocytes by B16F10 melanoma cells there is a rapid increase in acid sphingomyelinase activity and ceramide production as compared to acid sphingomyelinase-deficient thrombocytes or P-selectin-deficient thrombocytes. A lack of interaction of B16F10 melanoma cells and thrombocytes was excluded by activation of PLCγ, JNK and MAP kinase, indicating that these signaling events are stimulated in both, wild-type and P-selectin-deficient platelets, proving that B16F10 melanoma cells interact with and activate P-selectin-deficient thrombocytes.

The molecular mechanisms of tumor metastasis are currently fairly incomplete, though metastasis plays a crucial clinical role in cancer patients. Acid sphingomyelinase is iidentified as a novel target molecule for the inhibition of tumor metastasis. In order to pharmacologically inhibit the thrombocytic P-selectin system, an intravenous injection of fucoidan showed a decrease of tumor metastasis of B16F10 melanoma cells by approximately 75%. This indicates that tumor metastasis can be blocked pharmacologically, which is of great clinical interest.

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List of Publications

Rompaey LV, Potter M, Adams C, Grosveld G. **Tel induces a G1 arrest and suppresses Ras-induced transformation.** Oncogene. 2000 Nov 2;19(46):5244-50.

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Kirschnek S, Adams C, Gulbins E. **Annexin II is a novel receptor for Pseudomonas aeruginosa. Biochem Biophys Res Commun.** 2005 Feb 18;327(3):900-6.

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Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Fachbereiche 6 und 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Molecular Mechanisms of Hematogenous Tumor Metastasis“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Constantin Adams befürworte.

Essen, den 11. Juni 2007

Prof. Dr. Erich Gulbins

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Fachbereiche 6 und 9 zu Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 11. Juni 2007

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Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 und 9 zu Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den 11. Juni 2007

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