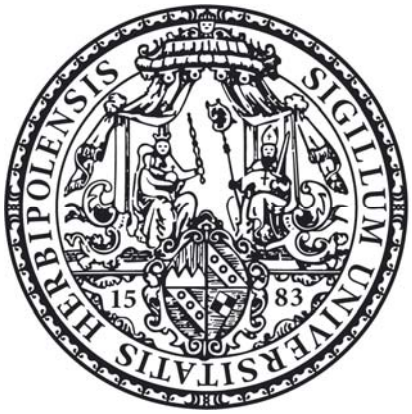

Mechanisms and functions of the mast cell-activated contact system in inflammatory reactions

Mechanismen und Funktionen des Mastzell-aktivierten Kontaktsystems für Entzündungsreaktionen

Doctoral thesis for a doctoral degree
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1 SUMMARY

Mast cell activation in allergic and inflammatory disease causes increased vascular permeability and edema. This thesis identifies a paracrine mechanism, by which heparin released from intracellular granules, is involved in mast cell-evoked alteration of endothelial barrier function *in vivo*.

Negatively charged heparin initiated factor XII-driven contact activation. Activated factor XII triggered the formation of the inflammatory mediator bradykinin in plasma. Congenital deficiency and pharmacological targeting of factor XII and kinin B2 receptor provided protection from mast cell-heparin-induced leukocyte-endothelial adhesion and hypotension in rats and mice. Intravital laser scanning microscopy and tracer measurements showed that heparin increased leakage with fluid extravasation in skin microvessels in mice. Deficiency in factor XII or kinin B2 receptor conferred resistance to heparin-induced skin edema and largely protected mice from endothelial barrier dysfunction, caused by allergen-induced mast cell activation and anaphylactic reactions. In contrast, heparin and mast cell activation caused excessive edema formation in mice, deficient in the major inhibitor of factor XII, C1 esterase inhibitor. Hereditary angioedema patients, lacking C1 esterase inhibitor, suffered from allergen-induced edema. The data indicate that mast cell-heparin-initiated bradykinin formation plays a fundamental role in defective barrier function of pathological mast cell-mediated inflammation, hypotension and edema formation.

2 ZUSAMMENFASSUNG

Aktivierte Mastzellen sind bei Allergien und Entzündungskrankheiten an der Ödembildung beteiligt. Diese Arbeit zeigt, wie von aktivierten Mastzellen freigesetztes Heparin die Gefäßpermeabilität erhöht. Mastzell-Heparin aktiviert im Plasma den Blutgerinnungsfaktor XII. Aktiver Faktor XII startet die Bildung des Entzündungsmediators Bradykinin durch Plasmakallikrein-vermittelte Spaltung von hochmolekularem Kininogen. Bradykinin führt zur Ödembildung und Vasodilatation. Bei Ratten und Mäusen blockieren Faktor XII- oder Kinin B2 Rezeptor-Inhibitoren die Heparin-induzierte und Bradykinin-vermittelte Leukozyten-Endothel Adhäsion und den arteriellen Blutdruckabfall. Um den Heparin-vermittelten Flüssigkeitsaustritt aus Mikrogefäßen in der Haut von Mäusen zu analysieren, wurde eine intravitale konfokale Mikroskopietechnik etabliert. Genetische Inaktivierung oder pharmakologische Blockierung von Faktor XII oder Kinin B2 Rezeptoren hemmen den Heparin-vermittelten Flüssigkeitsaustritt. Sowohl in systemischen als auch in cutanen passiven Anaphylaxiemodellen waren Faktor XII- und Kinin B2 Rezeptor-defiziente Mäuse vor Allergen-induzierten Ödemen und anaphylaktischen Reaktionen geschützt. Im Gegensatz dazu führte eine Allergenexposition zu einer überschiessenden Ödembildung in C1 Esterase Inhibitor-defizienten Mäusen, bei denen das Gen für den wichtigsten Inhibitor von Faktor XII inaktiviert wurde. Bei Hereditären Angioödem-Patienten, denen funktionaler C1 Esterase Inhibitor fehlt, lösen allergischen Reaktionen Ödemattacken aus.

Zusammenfassend zeigen diese Daten erstmals, dass die durch Heparin gestartet Bradykinin-Bildung, eine bedeutende Rolle bei der fehlerhaften Barrierenfunktion der pathologischen Mastzell-vermittelten Entzündungen, Blutdruckabfall und Ödembildung spielt.

3 INTRODUCTION

3.1 The contact system

The plasma contact system comprises the serine protease zymogen factor XII (FXII), plasma prekallikrein (PPK), the cofactor high molecular weight-kininogen (HK) and C1 esterase inhibitor (C1INH), the major inhibitor of activated FXII (FXIIa) and plasma kallikrein (PK). The name contact system is due to FXII-binding to negatively charged surfaces. “Contact” to surfaces induces an autoactivation in FXII resulting in small amounts of FXIIa that convert PPK into its active form PK. In a feedback loop PK activates additional FXII, which amplifies the initial signal (Cochrane and Revak, 1980, Figure 1).

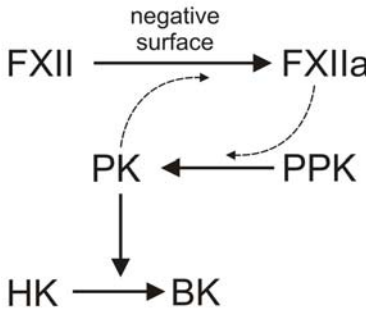

Figure 1. The plasma contact system. FXII is autoactivated to FXIIa that cleaves PPK to form PK. PK enhances FXIIa formation in a positive feedback loop. PK cleaves HK to liberate BK.

FXIIa triggers four plasma cascade pathways: the intrinsic pathway of coagulation, the fibrinolytic, the complement and the kallikrein-kinin system (Margolis, 1958; Ratnoff et al., 1961; Kaplan and Austen, 1972; Ghebrehiwet et al., 1981; Muller and Renne, 2008). The kallikrein-kinin system generates vasoactive proinflammatory kinin hormones. The peptide-hormone bradykinin (BK) is released by limited proteolysis from its precursor HK by PK action. PK cleaves HK at the C-terminus of the BK sequence, leaving the BK sequence attached to the C-terminal end of the HK-heavy chain (Nishikawa et al., 1992). Then a second cleavage liberates BK from the HK-heavy chain and a two-chain, kinin-free HK is formed. The two-chain from consists of the heavy chain (65 kDa) that is disulfide bond linked to the light chain (56 kDa) (Thompson et al., 1978). The vasoactive proinflammatory BK is a nonapeptide with the amino acid sequence Arg^{1}-Pro^{2}-Pro^{3}-Gly^{4}-Phe^{5}-Ser^{6}-Pro^{7}-Phe^{8}-Arg^{9}. It binds to the cell surface kinin B2 receptor (B2R) with a dissociation constant K_D = 1.2 nM (Schremmer-

Danninger et al., 1995; Yang et al., 1999). B2R belongs to the G protein-coupled receptor family, which are characterized by seven transmembrane-spanning helices. B2R is predominantly coupled to G proteins of the $G\alpha_q$ and $G\alpha_i$ families (Liao and Homcy, 1993; Yang et al., 1999). B2R is constitutively expressed in various cell types, such as endothelial cells, vascular smooth muscle cells and cardiomyocytes (Shukla et al., 2006). BK binding to B2R increase intracellular calcium levels, that trigger activation of cyclic adenosine monophosphate/cyclic guanosine monophosphate (cAMP/cGMP)-dependent protein kinases (PKA, PKG) and protein kinase C (PKC) (Newton, 1995; Francis et al., 2010). A common target for all three protein kinases is the actin-binding vasodilator-stimulated phosphoprotein (VASP) (Butt et al., 1994; Chitaley et al., 2004), which is localized to cell-cell contacts and involved in actin dynamics (Reinhard et al., 1992). The actin cytoskeleton is linked to interendothelial junctions, which connect endothelial cells to each other (Drenckhahn and Franz, 1986; Rimm et al., 1995). Actin assembly and disassembly regulates the integrity of these cell-cell adhesions and therefore the endothelial cell permeability (Stevenson and Begg, 1994; Anderson and Van Itallie, 1995). One type of interendothelial junctions is tight junctions. A tight junction-associated scaffold protein is αII-spectrin, which binds to VASP, linking the plasma membrane with the actin cytoskeleton. Complexes of VASP with αII-spectrin stabilize cell-cell contacts (Benz et al., 2008). VASP phosphorylation by PKA/PKG or PKC inhibits the interaction to spectrin leading to destabilization of cell-cell contacts, disassembly of tight junction, formation of cellular gaps, followed by increase in vascular permeability (Comerford et al., 2002; Benz et al., 2008). Another BK-triggered signaling pathway mediates the rearrangement of actin filaments into stress fibers, resulting in cellular contraction, which also induces tight junction disassembly and increased permeability (Ma and Xue, 2010, Figure 2).

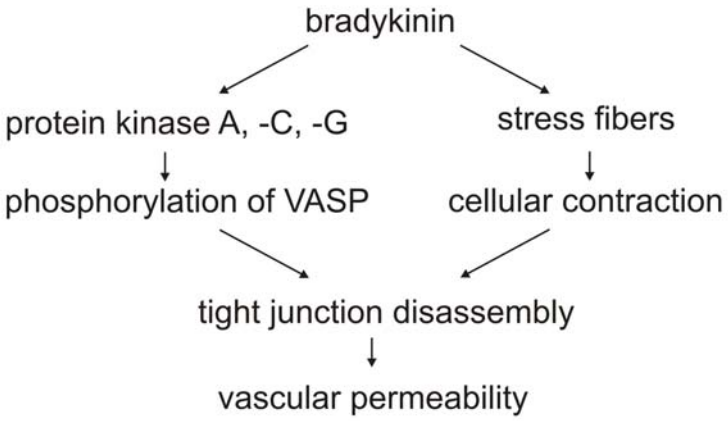

Figure 2. Bradykinin signaling. Bradykinin binding to its B2 receptor activates protein kinase A, C, and G, which phosphorylate VASP. Bradykinin induces stress fiber formation and cellular contraction leading to tight junction disassembly and increase of vascular permeability.

After BK binding to B2R the receptor is internalized (Pizard et al., 1999), and continues signaling. Non-receptor bound BK has a half-life of <1 min in plasma (Frick et al., 2006) and is degraded by endo- and exopeptidases, such aminopeptidase P, carboxypeptidase M and N and neutral endopeptidase (Skidgel, 1992). The major BK-degrading enzyme is the angiotensin-converting enzyme (ACE), also known as kininase II. It chips off the C-terminal dipeptide (Phe^8-Arg^9) from BK and is therefore classified as an exopeptidase (peptidyl dipeptidase). The resulting BK-(1-7) does not bind to B2R (Kokkonen et al., 2000).

3.2 Coagulation factor XII

FXII is a glycoprotein of 80 kDa that is primarily produced by hepatocytes. It circulates in plasma as a single chain zymogen with a concentration of 30-35 µg/mL (0.37 µM). The zymogen consists of 596 amino acids (Fujikawa and McMullen, 1983; McMullen and Fujikawa, 1985). FXII is composed of a fibronectin domain type II (at the N-terminus), an epidermal-growth-factor-like domain, a fibronectin domain type I, a second epidermal-growth-factor-like domain, a kringle domain, a proline-rich region and the catalytic domain (McMullen and Fujikawa, 1985). The catalytic domain with the

active site of FXIIa contains the catalytic triad Asp^{442}, His^{393}, and Ser^{554} (Schloesser et al., 1997).

FXII is activated to FXIIa (α-FXIIa) via autoactivation or proteolytic cleavage by plasma proteinases including PK and plasmin. The peptide-bond connecting Arg^{353}-Val^{354} is cleaved and a two-chain molecule is generated. The heavy chain (353 amino acids, 50 kDa) and the light chain (243 amino acids, 30 kDa) are hold together by a single disulfide bond between Cys^{340}-Cys^{346}. FXII-autoactivation takes place upon contact to negatively charged surfaces resulting in conformational changes (Samuel et al., 1992). The binding of zinc ions to FXIIa results in an increased susceptibility to autoactivation and stabilizes certain conformations in the activation reaction (Bernardo et al., 1993). FXIIa cleavages at the peptide bonds Arg^{334}-Asn^{335} and Arg^{343}-Leu^{344} by PK separate the heavy from the light chain. The resulting activated FXII-fragment (β-FXIIa, FXIIf, 28 kDa) consists of the light chain and nine amino acids of the C-terminal part of the FXII heavy chain (Dunn and Kaplan, 1982; Tankersley and Finlayson, 1984). Although retaining its proteolytic activity towards PPK, β-FXIIa is unable to bind to negatively charged surfaces and therefore is not able to convert factor XI (FXI) to active FXI (FXIa) and fails to promote blood clotting (Dunn et al., 1982).

The major inhibitor of α- and β-FXIIa, and PK in plasma is C1INH (de Agostini et al., 1984; Pixley et al., 1985b). C1INH is a suicide serine protease inhibitor (serpin) that inhibits proteases from the coagulation pathway and also the complement protease C1s and C1r. C1INH is a highly glycosylated protein of 478 amino acids with a molecular weight of 94 kDa (Zuraw and Curd, 1986). C1INH consists of an N-terminal domain of 113 amino acids, and a serpin domain of 365 amino acids, both are connected via two disulfide bonds (Bos et al., 2002). The serpin contains a reactive, flexible peptide loop for interaction with target proteases. Essential residues of this loop are called P1–P1´ residues, which are located next to each other (Bos et al., 2002). Cleavage of this specific peptide-bond in the reactive center of the serpin (exon 8 codes for the reactive center, (Nielsen et al., 1998) triggers a molecule-rearrangement

and results in the formation of a covalent peptide-bond between the P1 residue of the C1INH and the Ser^{554} from the active site of the protease (Davis, 2008). The formed peptide-bond between C1INH and FXIIa inactivates the catalytic triad of the protease and the protease is inhibited. Other physiological inhibitors of FXIIa comprise antithrombin and α_2-macroglobulin (Pixley et al., 1985a; Berrettini et al., 1989).

3.3 Activators of kallikrein-kinin system

Formation of BK can be initiated by several FXII activators. A common feature of all known activators is that they are negatively charged macromolecules, which can be divided into two groups: artificial substances such as glass, kaolin and ellagic acid and natural occurring substances including nucleotides, sulfates, micelles and glycosaminoglycans (Table 1).

Table 1. Contact system activators. (Maas et al., 2011)

Artificial activators	Natural activators
Glass	DNA/RNA
Kaolin	Sulfatides/micelles
Celite and cilica	Urea crystal
Ellagic acid	Collagens
Polyethylene	Glycosaminoglycans
Dextran sulfate	Endotoxin
	Polyphosphates
	Misfolded proteins

Glycosaminoglycans such as squid chondroitin sulfate E and heparin from rat peritoneal mast cells activate the contact system *in vitro* (Hojima et al., 1984). In human plasma *ex vivo* experiment heparin from a murine mastocytoma-derived mast cell line accelerated the reciprocal activation of FXIIa and PK and the autoactivation of FXII (Brunnee et al., 1997). Recent studies identified new FXII activators: misfolded protein-aggregates activate FXII leading to activation of the kallikrein-kinin system without inducing the intrinsic pathway of coagulation (Maas et al., 2008). In contrast, platelet-derived polyphosphates activate the contact system resulting in activation of both, the intrinsic pathway of coagulation and the kallikrein-kinin system (Muller et al., 2009). The

kallikrein-kinin system can be activated by bacteria. The components of the contact system assembly on structures of the bacterial surface (negatively charged lipopolysaccharides and lipid A of *Escherichia coli (E. coli)*; peptidoglycan and teichoic acid of *Staphylococcus aureus;* fimbriae of *Salmonella typhimurium* and M protein of Group A *streptococci*) (Kalter et al., 1983; Ben Nasr et al., 1996; Persson et al., 2000; Mattsson et al., 2001; Frick et al., 2007). On the bacteria surface HK is cleaved by bacterial secreted kininogen-degrading proteinases to release BK (streptococcal proteinase is secreted by Group A *streptococci*; *Staphylococcus aureus* and *Streptococcus pyogenes* secrete a cysteine proteinase (Herwald et al., 1996; Imamura et al., 2005; Linder et al., 2010)) . Since it was shown, that PPK can be activated FXII-independently on endothelial cells (Motta et al., 1998) other substances were discovered that activate the kallikrein-kinin system FXII-independently. Intracellular chaperon heat shock protein 90 (Joseph et al., 2002), the mitochondrial prolylcarboxypeptidase (Shariat-Madar et al., 2004) and extracellular carbonicanhydrase (Gao et al., 2007) have the capacity of activating PPK. Physiological importance of these activators is currently unknown.

3.4 Mast cells

Mast cells (MC) are derived from multipotent hematopoietic progenitor cells in the bone marrow (Kitamura et al., 1981). The cells enter into the blood circulation and differentiate within various tissues (Hallgren and Gurish, 2007). Migration and differentiation take place under the influence of the local micromilieu, including growth factors, integrins, chemokines and adenosine nucleotides (Taub et al., 1995; McCloskey et al., 1999). The most important growth factor for MC is stem cell factor, the ligand for the c-kit receptor (Li and Krilis, 1999).

Two major subtypes of human MC can be distinguished dependent on the presence of tryptase ($_T$) or tryptase and chymase ($_{TC}$): MC_T and MC_{TC} (Moon et al., 2010). MC_T are

the prominent MC type in the mucosa of the respiratory and gastrointestinal tract and increase with mucosal inflammation. MC_{TC} are localized within connective tissues, such as the dermis, submucosa of the gastrointestinal tract, heart, conjunctiva, and perivascular tissues (Metcalfe, 2008). In rodents connective tissue MC (MC_{CT}) and mucosal MC (MC_M) are described. MC_{CT} are predominantly found in the skin and peritoneal cavity. Their granules contain the polysaccharide heparin, histamine and carboxypeptidase A. In contrast, MC_M are found predominantly in the mucosal layer of the gut and lungs. Their granules contain chondroitin sulfate as the major polysaccharide compound and relatively less histamine and carboxypeptidase A (Heib et al., 2008; Rao and Brown, 2008). In human and mice the different milieu of cytokines and the tissue-specific expression of stem cell factor are responsible for the heterogeneity of differentiation and distribution of MC in specific tissues.

MC can be activated by different mechanisms: via toll-like receptors, which are important for direct pathogen recognition; via complement products such as C3a and C5a (Nilsson et al., 1996; Marshall, 2004; Dawicki and Marshall, 2007); and via IgG-antibody, which binds to its high-affinity receptor FcγRI. The best characterized pathway for MC activation is the antigen-dependent aggregation of the high-affinity receptor for IgE (FcεRI). FcεRI contains three immunoreceptor tyrosine–based activation motifs (ITAM), which are phosphorylated after receptor aggregation. The phosphorylations trigger a number of downstream signaling events, such as activation of phospholipase C-γ, increase of intracellular calcium and PKC activation, activation of transcription factors and metabolism of arachidonic acid via phospholipase A_2. Phosphatidylinositol kinase (PI3K) is activated, that regulates the formation of lipid mediators, such as spingosine 1-phosphate, that in turn regulates MC degranulation. Soluble N-ethylmaleimide attachment protein receptors complexes are formed, that promote membrane fusion and thereby mediator secretion (Blank et al., 2002; Kalesnikoff and Galli, 2008; Stone et al., 2010). MC produce an array of vasoactive mediators and proinflammatory substances that are either preformed granule-

associated mediators, newly generated lipid mediators or belong to a wide variety of cytokines and chemokines (Kalesnikoff and Galli, 2008). Preformed mediators include histamine (Riley and West, 1953), proteoglycans, serine proteases (tryptase and chymase, (Kido et al., 1985), carboxypeptidase A and antimicrobial peptides, which play roles in recruitment and activation of T cells, neutrophiles, basophiles and eosinophiles. *De novo* synthesized mediators include lipids, cytokines, and chemokines. Lipid mediators are prostaglandins, leukotrienes and platelet-activating factor, which contribute to recruitment and activation of monocytes and macrophages (Liu et al., 2010; Stone et al., 2010). A well-established concept is that MC mediators, particularly the vasoactive amines histamine or serotonin, increase capillary leakage and may rapidly produce edema. Histamine receptor antagonists are therapeutically used to treat edema formation and allergic reactions associated with aberrant MC activity, e.g. in asthmatic disease (Theoharides and Kalogeromitros, 2006). MC secretory granules also contain highly sulfated polysaccharides, such as heparin. Although heparin is one of the most studied molecules, its physiological function *in vivo* has yet to be determined (Oschatz et al., 2011).

3.5 Heparin

Heparin is a linear, unbranched, highly sulfated polysaccharide consisting of repeating disaccharide units of 1-4-linked iduronic acid and glucosamine. It is exclusively stored in cytoplasmatic secretory granules of MC_{TC} (Casu, 1985; Rabenstein, 2002). The average heparin disaccharide unit contains 2.7 sulfate groups. Therefore heparin has a high negatively charge density (Capila and Linhardt, 2002, Figure 3).

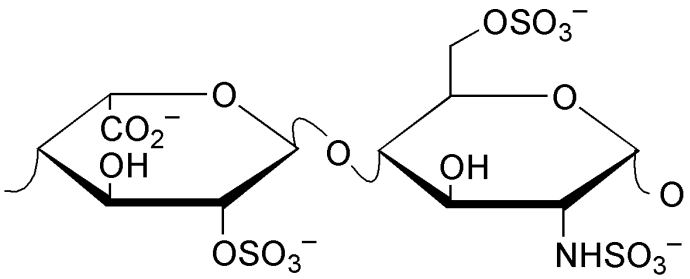

Figure 3. Heparin disaccharide unit. The average heparin disaccharide consists of iduronic acid and glucosamine and contains 3 sulfate groups (Rabenstein, 2002).

Heparin is biosynthesized in the Golgi apparatus as heparin proteoglycan (750–1000 kDa), which consists of a serglycin core protein to which multiple heparin polysaccharide chains (600–100 kDa) are covalently attached (Robinson et al., 1978). Biosynthesis of polysaccharide chains takes place in three phases: chain initiation, polymerization and polymer modification. The first step involves the attachment of a linkage-region tetrasaccharide to a serine residue of the core protein (Salmivirta et al., 1996). Then, the heparin polysaccharide chains are built up by alternating addition of 1-4 linked iduronic acid or glucosamine units to the tetrasaccharide core. Approximately 150 disaccharide units are added before the synthesis terminates (Capila and Linhardt, 2002). During elongation the chain is modified by a series of sequential N-deacetylase/N-sulfotransferase, epimerase and O-sulfotransferase-catalyzed reactions (Lindahl et al., 1972; Jacobsson and Lindahl, 1980; Rosenberg et al., 1997). All subsequent modifications are dependent on the N-sulfate groups for substrate recognition, which makes the N-deacetylation/N-sulfation step very important. The structural variability of the heparin polymer is the result of incomplete modifications by the biosynthetic enzymes. Finally the polysaccharide chains are randomly cleaved along the chains to fragments of 5-40 kDa. Heparin is stored in cytoplasmatic secretory granules of MC_{TC} as non-covalent complexes with histamine, MC proteases and other mediators (Rabenstein, 2002). The physiological significance of heparin in MC granula for storage of other mediators has been shown by disruption of the *N-deacetylase/N-sulfotransferase* gene in mice (Forsberg et al., 1999; Humphries et al., 1999). Targeting the modifying enzyme blocked the biosynthesis of “normal” (sulfated) heparin, resulting in largely decreased amounts of several MC proteases and histamine. MC-released

heparin can interact with a number of biologically important proteins (growth/differentiation factors such as fibroblast growth factor-1 and -2, cytokines such IL-8, protease inhibitors such as antithromin III (ATIII), enzymes or adhesion molecules such as selectin or fibronectin, (Capila and Linhardt, 2002; Sugahara and Kitagawa, 2002). Thereby heparin plays an essential role in the regulation of various physiological processes including inhibition of complement activation (Weiler et al., 1992), inhibition of angiogenesis (Hasan et al., 2005) and tumor growth (Borsig et al., 2001).

Heparin is used as an anticoagulant drug based on its ability to accelerate the rate at which ATIII inhibits serine proteases in the blood coagulation cascade. ATIII by itself is a relative weak inhibitor. But heparin-ATIII binding via a unique pentasaccharide sequence enhances ATIII mediated inhibition of thrombin and activated factor X (FXa) (Lindahl et al., 1979; Rosenberg and Lam, 1979). The ATIII-binding pentasaccharide sequence is rare and occurs only in one third of the heparin chains. Its most distinguishing feature is an unusual 3-O-sulfate group on an internal glucosamine residue, which is essential for its high affinity to ATIII (Lindahl et al., 1980; Petitou et al., 1988; Walenga et al., 1988).

3.6 Hereditary angioedema type III

Patients with hereditary angioedema (HAE) suffer from life-threatening edema, which can lead to death. HAE has a prevalence of 1:10.000 to 1:50.000. Affected individuals are from all races. HAE is an autosomal dominant disease associated with episodic attacks of subcutaneous or submucosal edema that affected extremities, face or larynx (Frank et al., 1976; Davis, 2008; Zuraw, 2008). Three different types of HAE exist. HAE I and II are caused by mutations in the *Serping1* gene coding for the C1INH protein. Type I is caused by mutations leading to decreased levels of C1INH (quantitative defect). In type II the mutated C1INH protein is secreted in normal concentration but has a misfolded formation resulting in reduced enzymatic activity (functional defect). In

HAE type I and II, absence of C1INH function leads to excessive BK generation, resulting in angioedema. Recently, a new type of HAE (HAE type III) has been identified, which clinically does not differ from HAE I or II. The disease exhibits no quantitative or functional C1INH abnormalities. Most affected individuals are women (Bork et al., 2000; Bork et al., 2006). Swelling attacks are often during pregnancy or when receiving oral contraceptives containing oestrogens (Binkley and Davis, 2000; Martin et al., 2001; Gupta et al., 2004; Bork et al., 2006; Vitrat-Hincky et al., 2010). Genetic analyses revealed two missense mutations at position Thr328 (1032C→A: Thr exchange to Lys and 1032C→G: Thr exchange to Arg) in the *factor 12* gene of affected women from different families as a possible cause of HAE III (Cichon et al., 2006; Dewald and Bork, 2006; Picone et al., 2010). Biochemical studies showed that FXII-Thr328Lys mutation is a gain-of-function mutation, which increases FXII enzymatic activity but do not alter FXII plasma levels (Cichon et al., 2006). Enhanced FXII enzymatic activity leads to enhanced kinin production. Stimuli triggering the periodic episodes of excessive vascular leakage are poorly defined.

3.7 Aim of the study

The biochemistry of the contact system is well characterised, however it is less understood how the system is activated *in vivo*. Heparin is a high negatively charged polysaccharide that is exclusively found in MC granules (Forsberg et al., 1999). It is known, that MC-heparin activates the contact system *in vitro* leading to BK formation (Hojima et al., 1984; Brunnee et al., 1997). Heparin is used as an anticoagulant drug. In rare cases, adverse reactions including drop in blood pressure and edema in various tissues including the skin have been reported after heparin application (MacLaughlin et al., 2002). Although heparin is a well studied molecule, it is not known whether heparin functions in MC-triggered vascular effects *in vivo*.

The aim of this study was to analyze MC-heparin for its ability to trigger activation of contact system *in vivo* using vascular leakage and anaphylaxis models in rodents.

The goals of the presented work can be divided into the following parts:

I) Characterisation of MC-heparin as a FXII-dependent activator of the contact system *in vitro*

II) Analysis of MC-heparin-driven BK-mediated vascular leakage and hypotension in rodents

III) Analysis of genetically and pharmacologically targeting of FXII or B2R for MC-driven effects

IV) Investigation of allergen-stimulated MC to induce BK-mediated edema in genetically altered mice and in hereditary angioedema patients

4 MATERIAL AND METHODS

4.1 Plasma and animals

4.1.1 Human plasma

FXI-, FXII-, and PK-deficient human plasma was purchased from George King Bio Medical (Overland Park, Kansas, USA). Control citrated plasma was collected from healthy volunteers (University Hospital Würzburg, Germany and blood transfusion unit of the Karolinska Hospital, Stockholm, Sweden).

4.1.2 Animals

All procedures and animal studies were approved by local authorities. Wild-type (WT) C57BL/6 mice and male Spraque-Dawley rats (8 weeks of age) were purchased from Charles River Wiga (Sulzfeld, Germany) (Oschatz et al., 2011). MC deficient B6.Cg-*Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ mice (*Kit*$^{W\text{-}sh/W\text{-}sh}$) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) (Oschatz et al., 2011). Generation and phenotyping of *Serping1*$^{-/-}$ mice is described under 4.2.9. All used mice strains are listed below (Table 2). Mice were used between 6-12 weeks of age (Oschatz et al., 2011).

Table 2: Genetically altered mice strains.

Mice strain	Name of inactivated protein	Reference
F12$^{-/-}$	Factor XII	(Pauer et al., 2004)
Bdkrb2$^{-/-}$	Kinin B2 Receptor	(Borkowski et al., 1995)
Kit$^{W\text{-}sh/W\text{-}sh}$	C-Kit	(Yamazaki et al., 1994)
Serping1$^{-/-}$	C1 esterase inhibitor	(Oschatz et al., 2011)
VASP$^{-/-}$	VASP	(Hauser et al., 1999)

4.2 Methods

4.2.1 Sodium dodecyl sulfate-polyacrylamide gelelectrophoresis and Western blotting

Protein/plasma samples were separated by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) using 10% or 12% gels (Table 3).

Table 3: Solutions for preparing SDS-PAGE gels.

	Stacking gel	Separating gel	
		10%	12%
H_2O	3.4 mL	4.0 mL	3.3 mL
30% acrylamide mix (Roth)	0.83 mL	3.3 mL	4.0 mL
1.5 M Tris (pH 8.8; Roth)	-	2.5 mL	2.5 mL
1.0 M Tris (pH 6.8; Roth)	0.63 mL	-	-
10% SDS (Roth)	0.05 mL	0.1 mL	0.1 mL
10% ammonium persulfate (Sigma)	0.05 mL	0.1 mL	0.1 mL
N,N,N′,N′-Tetramethylethylenediamine (Roth)	0.005 mL	0.004 mL	0.004 mL
Total volume	5 mL	10 mL	10 mL

Plasma samples were diluted in SDS-PAGE sample buffer (50 mM TrisHCl pH 6.8 (Roth, Karlsruhe, Germany), 100 mM dithiothreitol (Sigma, Steinheim, Germany), 2% SDS (Biotium/VWR international, Stockholm, Sweden), 0.1% bromophenol blue (Sigma), 10% glycerine (Roth)), heated to 95°C for 5 min and were loaded on the gel. As molecular weight marker Page Ruler prestained Protein Ladder (Fermentas, St.Leon-Rot, Germany) was used. Electrophoresis started with 80 V and voltage was increased to 150 V after samples reached the separation gel border (electrophoresis buffer: 25 mM Tris (Roth), 250 mM glycerine pH 8.3, 0.1% (w/v) SDS). Proteins were transferred (0.65 mA/ membrane cm^2 for 1.5 h at room temperature (RT), transfer buffer: 39 mM glycerine, 48 mM Tris base, 0.037% SDS, 20% methanol (Sigma)) to a in methanol activated polyvinylidene difluoride membrane (pore size 0.45 µm, Milipore, Schwalbach, Germany) using a biometra Power Pack P25 power supply system (Göttingen, Germany). The nitrocellulose was stained with Ponceau S (Roth) to verify protein presence. The membrane was washed with PBS-Tween (PBS-T; PBS with 0.05% (v/v) Tween 20, Roth). To reduce unspecific antibody (Ab) binding, membrane was blocked with 5% (w/v) milk powder (Roth) in PBS-T for 2 h at RT or overnight at 4°C. Membrane was incubated with diluted primary Ab in 5% milk powder in PBS-T (Table 4) for 2 h at RT, washed twice with PBS-T, and incubated with diluted secondary Ab in 5% milk powder in PBS-T (Table 4) for 1 h at RT. Membrane was washed twice with PBS-T and proteins were detected with Amersham ECL Western

Blotting Detection reagents (GE healthcare, Freiburg, Germany), X-ray films from Thermo scientific (Rockford, Illinois, USA) and a developer machine (Eastman Kodak Company, Rochester, New York, USA) according to the manufacturer's instructions.

Table 4: Antibodies for Western blotting.

Primary antibody	Concentration /Dilution	Secondary antibody	Dilution	Company/ Reference
PK6, purified mouse mAb to heavy chain of PK	2 µg/mL	goat α-mouse (Jackson Immuno Research; 115-035-003)	1:2000	(Hock et al., 1990)
HKL12, purified mouse mAb to light chain of HK	0.5 µg/mL	goat α-mouse (Jackson Immuno Research; 115-035-003)	1:2000	(Renne et al., 2005b)
I108, purified sheep pAb to heavy chain of HK	1 µg/mL	donkey α-sheep (Jackson Immuno Research; 713-035-147)	1:5000	
α-HK AS94, sheep pAb to light and heavy chain of HK	1:500 Serum	donkey α-sheep (Jackson Immuno Research; 713-035-147)	1:2000	
I107, purified sheep pAb to light chain of HK	1 µg/mL	donkey α-sheep (Jackson Immuno Research; 713-035-147)	1:2000	(Renne and Muller-Esterl, 2001)
MBK3, purified mouse mAb to BK sequence in HK	1 µg/mL	donkey α-rabbit (Jackson Immuno Research; 711-035-1520)	1:2000	(Haasemann et al., 1991)
GAHu/FXI, goat pAb to FXI	1:2500 Serum	donkey α-goat (Jackson Immuno Research; 705-035-003)	1:2000	Nordic Immunological Laboratories, Tilburg, the Netherlands
GAHu/FXII, goat pAb to FXII	1:2500 Serum	donkey α-goat (Jackson Immuno Research; 705-035-003)	1:2500	Nordic Immunological Laboratories, Tilburg, the Netherlands
α-C1INH, purified rabbit pAb to 23 -36 amino acid of murine C1INH	2 µg/mL	donkey α-rabbit (Jackson Immuno Research; 711-035-1520)	1:3000	(Oschatz et al., 2011)

Horseradish peroxidase-conjugated secondary Ab were from Jackson Immuno Research (Hamburg, Germany).

4.2.2 Isolation of heparin from mast cells

Male rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.; Nembutal, Ceva, Paris, France) and 20 mL of sterile ice-cold PBS were injected i.p.. After 1 min peritonealexudate was taken. MC were isolated via two centrifugation steps at 400 x *g* for 15 min at RT using a density gradient of 22.5% w/v Metrizamide (Sigma). MC were resuspended in PBS, centrifuged and 750 µL RPMI-media (Life Technologies, Lohne, Germany) were added. MC concentration was determined by Giemsa (Roth)-stained cytospots to 3.5 x 10^5 cells per mL. MC suspension was halved and incubated with compound 48/80 (C48/80; Simga; 4 µg/mL), or medium for 45 min at 37°C in a 5% (v/v) CO_2 atmosphere. Subsequently, the supernatant was separated by centrifugation at 2000 × *g* for 15 min at 25°C and incubated for 1 h at 37°C with protease inhibitors (1 mM EDTA (Sigma), 1 mM phenylmethylsulfonylfluorid (Roth), 1 mM leupeptin (Sigma), 5 mM benzamidine (Roth), 1 mM soybean trypsin inhibitor (Roth) and 10 mg/mL Pefabloc (Roth)). All low molecular molecules of the supernatant (<2000 Da) were removed by a two-time gel filtration on a Sepharose 4B column (Bio-Rad, Hercules, California, USA) and the passage was lyophilized.

The purity of MC-derived glycosaminoglycans was determined as previously described (Katz et al., 1986; Oschatz et al., 2011). Heparinase (from *Flavobacterium heparinu*) and chondroitinase ABC (from *Proteus vulgaris*) (both from Seikagaku, Tokyo, Japan) digests and absorbance measurements at 232 nm indicated that the purified material was >95% heparin (Oschatz et al., 2011). The amount of glycosaminoglycans was quantified by determining the uronic acid content using the carbazole method (Oschatz et al., 2011). Incubation of MC-purified heparin with trypsin, chymotrypsin, elastase, or papain (Sigma) had no measurable effect on the filtration behavior on Sepharose 4B columns (Bio-Rad) compared with untreated material, indicating that the size of the native molecule was not changed and that the glycosaminoglycan was not complexd with proteins (Oschatz et al., 2011).

4.2.3 Contact activation system assays *in vitro*

HK cleavage was initiated in fresh human citrate plasma using MC-derived heparin, heparinase treated MC-derived heparin, heparan sulfate (Sigma), dextran sulfate (Sigma), sulfated heparin or desulfated heparin. Following incubation for 30 min at 37°C, the reaction was stopped by addition of SDS-PAGE sample buffer containing 8% (m/v) SDS, and 0.25 µL plasma was separated by a 10% polyacrylamide gel and analyzed by Western blotting. Ab against BK MBK3 was used to detect uncleaved HK. To test the factors, which are involved in HK cleavage and BK liberation, FXI-, FXII-, and PK-deficient plasma or normal plasma were incubated with 20 µg/mL MC-derived heparin for 30 min at 37°C. Plasma was separated by SDS-PAGE and analyzed by Western blotting. To detect FXII, FXI, PK or low molecular weight-kininogen (LK) GAHu/FXII, GAHu/FXI, PK6 or I107 Ab were used, respectively.

BK concentrations were determined by MARKIT-M-Bradykinin ELISA according to the manufacturer's instruction (Dainippon Pharmaceutical, Osaka, Japan) (Oschatz et al., 2011).

4.2.4 Bradykinin generation on endothelial cell surfaces

Cultivation of EA.hy926 endothelial cells (Benz et al., 2008) and cell-binding assays with [^{125}I]-radiolabeled HK was performed as described previously (Renne et al., 2000; Oschatz et al., 2011). BK concentrations were determined by MARKIT-M-Bradykinin ELISA.

4.2.5 Coagulation assays

Adult mice were killed by inhalation of CO_2. 800 µL of citrate anticoagulant blood (0.36% citrate) were taken by cardial puncture. Blood was centrifuged at 5.000 rpm for 5-10 min at RT. Supernatant was taken and centrifuged again at 5.000 rpm for 5-10 min at RT to obtain platelet pure plasma (PPP). To prepare an activated partial thromboplastin time (aPTT)-standard curve for unfractionated heparin (Liquemin,

Roche, Mannheim, Germany), 0-2 µg/mL of the polysaccharide was added to murine PPP. APTT was measured by automated blood coagulation system (BCS XP 1.1, Siemens, Eschborn, Germany) with reagents according to the protocols of the manufacturer. To determine heparin levels in murine platelet pure plasma after systemic anaphylaxis, aPTT was measured and compared to the standard curve. Specificity of heparin anticoagulant activity was confirmed by repeated aPTT determination after pretreatment with protamine sulfate (Sigma; 1-2 µg/mL) or 0.5 U/mL heparinase (Sigma) for 30 minutes at 37 ºC (Oschatz et al., 2011).

4.2.6 Heparin-induced bradykinin generation in rats

Male rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.; Nembutal, Ceva, Paris, France). Catheters were placed into the carotid artery and *vena cava*, and the jugular artery and vein. The rats were placed in a supine position on a heated operating pad. Mean systolic arterial blood pressure was measured in the left carotid artery using an arterial catheter connected to a Statham pressure transducer (type P 23 I; Gould, Oxnard, California, USA) and recorder (LS 64; Linseis, Selb, Germany). A bolus of 10 mg/kg bw heparin was applied into the aorta or femoral artery to trigger hypotension or inflammation, respectively. Continuously the mean arterial blood pressure and vital signs of the animals during challenge were monitored. For some experiments, rats were treated with Icatibant (Jerini, Berlin, Germany; 100 µg/kg bw) prior to heparin challenge. Frozen cross-sections of the A*. femoralis* and V. *femoralis* 2 mm caudal to the knee joint were stained with Haematoxylin-Eosin (Sigma) 30 min after heparin application. Heparin-induced bradykinin generation in rats resulted from collaboration with B. Lecher and was published before in Oschatz et al., 2011.

4.2.7 Analysis of vascular leakage

4.2.7.1 Miles edema model

Mice were anesthetized by i.p. injection of Avertin (2,2,2-tribromoethanol and 2-methyl-2-butanol; Sigma; 15 µL/g bw of a 2.5% solution with PBS) (Oschatz et al., 2011). Evans blue (Sigma; 10 µL/g bw of a 0.25% solution with 0.9% NaCl; Braun, Melsungen, Germany) was injected intravenously in the retro-orbital. 5 min later, dorsal skin edema was induced by intradermal injections. To avoid irritation of the skin, hairs were griped with a forceps close to the root, slightly raised and set back to see the skin. For injection a single-use 1 mL syringe with a 30 G needle (unimed, Lausanne, Switzerland) was used. The needle tip was bent to a hook, gently punched into the skin and turned sideways to inject 50 µL of MC-heparin (1 mg/mL), C48/80 (50 µg/mL), NaCl, histamine (Sigma; 100 µM), BK (Sigma, 100 µM) or heparinase-treated MC-heparin (Miles and Miles, 1952). 10 mg/mL MC-heparin were incubated for 24 h at 25°C with 5 U heparinase (Sigma). Some WT mice were pretreated with H-D-Pro-Phe-Arg-chloromethylketone (PCK; Bachem, Bubendorf, Switzerland; 8 µg/g bw), Icatibant (100 µg/kg bw), or NaCl 5 min prior to MC-heparin injection. After 30 min, the mice were sacrificed, the skin was cut and tight attached on a flat surface. After 5-10 min the skin was photographed. The edema sites were excised using a circular template of 1.2 cm diameter. For edema with diameter >1.2 cm the entire blue area was excised by hand (Oschatz et al., 2011). The edema-skins were cutted in little pieces.

The Evans blue dye was extracted by incubation in 800 µL N,N-dimethylformamide (Sigma) overnight at 57°C (Donelan et al., 2006). After centrifugation at 20.000 rpm for 1 h, the supernatant was collected. On a 96-well plate (UV-star, Greiner bio-one, Stuttgart, Germany) the Evans blue fluorescence of 100 µL sample (double determination) was quantified photometrically (multiple reader Victor^2, PerkinElmer, Rodgau, Germany) at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. The concentration of Evans blue was normalized by dividing with the value of NaCl-induced signal in WT mice (set to 1.0).

4.2.7.2 Skin vascular leakage assay

To analyze vascular leakage in real time using intravital laser scanning microscopy, the method was used, which was originally described for leukocyte infiltration into vascular lesions (Eriksson et al., 2001; Oschatz et al., 2011). Mice were anesthetized by i.p. injection of Avertin (Oschatz et al., 2011). 3 ng/g bw FITC-dextran (150 kDa, Sigma) was injected into the retro-orbital plexus as tracer (Oschatz et al., 2011). Mice were placed on a cork plate in a supine position. A ventral skin window from the middleline of the abdomen to one side was excised and the skin was fixed plain next to the mice. Extravasation of tracer was started by topical application of MC-heparin (20 µL of a 1 mg/mL solution). After 1 min the drop was removed from skin with soft paper without touching the skin. Extravasation of tracer from microvessels (35–60 µm diameter) was visualized using a Nikon Eclipse E600 microscope equipped with a C1 laser scanning head and a 10x objective (Nikon, Düsseldorf, Germany) (Oschatz et al., 2011). Leakage was assessed for 3 min before and 30 min after topical application of MC-heparin (Oschatz et al., 2011). Tissue scans were performed at 30 sec intervals (Oschatz et al., 2011). Images were analyzed by EZ-C1 2.10 software (Nikon) and the intensity of extravasated tracer was quantified using ImageJ 1.34 NIH software (Oschatz et al., 2011).

4.2.8 Anaphylaxis models

4.2.8.1 Passive cutaneous anaphylaxis

Mice were anesthetized by i.p. injection of Avertin. To induce passive cutaneous anaphylaxis, mice were sensitized by intradermal injection of anti- dinitrophenyl IgE (α-DNP IgE; Sigma; 50 µL of a 1.5 µg/mL solution) or 50 µL steril 0.9% NaCl (control) in the dorsal skin (Oschatz et al., 2011). Intradermal injection was carried out as in the Miles edema model (Oschatz et al., 2011). After 20 h mice were challenged by retro-orbitally injection of dinitrophenyl-human serum albumin (DNP-HSA; Sigma; 7.5 mg/kg bw) together with Evans blue (10 µL/g bw of a 0.25% solution with 0.9% NaCl)

(Siebenhaar et al., 2008; Oschatz et al., 2011). After 30 min, the mice were sacrificed, the skin was removed and photographed. The entire edema site was excised by hand. The Evans blue dye was extracted by incubation in N,N-dimethylformamide overnight at 57°C (Donelan et al., 2006). After centrifugation at 20.000 rpm for 1 h, the supernatant was collected and the Evans blue fluorescence was quantified photometrically at an excitation wavelength of 620 nm and an emission wavelength of 680 nm.

4.2.8.2 Passive systemic anaphylaxis

For passive systemic anaphylaxis model, mice were injected with α-DNP IgE (1.25 µg/g bw) into the tail vene, after they were placed under a red light for 10 min (Oschatz et al., 2011). 24 h later mice were challenged by injection of 1 mg DNP-HSA (Sigma) into the tail vene (Oschatz et al., 2011). For some experiments arterial blood pressure was recorded by fluid-filled catheter connected to a pressure transducer (APT 300, Harvard Apparatus, March-Hugstetten, Germany) (Oschatz et al., 2011). In other cases 5-10 min after induction of systemic anaphylaxis animals were killed by Isofluran (Baxter, Unterschleißheim, Germany) and blood was taken by cardial puncture.

4.2.9 Generation of *Serping1*$^{-/-}$ mice

A DNA archive from ENU-mutagenised, C3HeB/FeJ male mice (Augustin et al., 2005) was screened for point mutations in the C1INH gene (*Serping1)* by polymerase chain reaction amplification of the respective chromosomal coding regions followed by heteroduplex analysis of the generated fragments using a temperature gradient capillary electrophoresis system. Fragments displaying typical heteroduplex migration patterns were sequenced to determine the nature of the nucleotide substitution. In one animal, a guanine (g) to adenine (a) transition at position +1 in the splice donor site of *Serping1* intron 2 was detected (Figure 4).

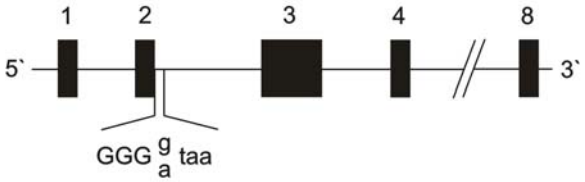

Figure 4. Schematic representation of the *Serping1* gene. Exons are shown as dark boxes. *Serping1*$^{-/-}$ mice have a single-base transition (g to a) at position +1 in the donor splice site of *Serping1* intron 2 (Oschatz et al., 2011).

Sperm from the mutant carrier animal was used for *in vitro* fertilization of C3HeB/FeJ WT oocytes and the embryos were transferred to foster females. The resulting pups were genotyped for the presence of the mutation and bred to produce homozygous progeny. A coupled reverse transcriptase-PCR on total RNA from the mutants showed that the mutation causes exon 2 to be removed during *Serping1* mRNA processing. In addition, elimination of exon 2 in the *Serping1* mRNA causes a frame-shift, which leads to the early termination of protein synthesis during translation. The generation of *Serping1*$^{-/-}$ mouse resulted from collaboration and was kindly provided by Reinhard Sedlmeier and was published before in (Oschatz et al., 2011).

For genotyping of *Serping1*$^{-/-}$ mice genomic DNA was isolated from mouse tails using a DNeasy blood & tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For PCR a Taq DNA Polymerase Kit (Qiagen) was used. PCR reactions were performed in total volume of 25 µL containing 2.5 µL Taq buffer, 5 µL Q-solution, 0.8 µM each of forward and reverse primer (forward primer C1Inh_1_for: 5´-CTGGCTCTGAGGCTAACTGG-3´; reverse primer C1Inh_1_rev: 5´-GAGAGGCTTCCCTCCTTCAC-3´; Eurofins, Ebersberg, Germany), 0.4 mM dNTP mix (Fermentas), 5 U Taq Polymerase and 50-100 ng DNA. The cycling condition were: preincubation at 94°C for 3 min, 59°C for 40 sec and 72°C for 1 min, 34 cycles of: denaturation step at 94°C for 45 sec, annealing step at 55°C for 45 sec and elongation step at 72°C for 45 sec. Final elongation step was at 72°C for 10 min. PCR fragments were separated by gelelectrophoresis on 1% agarose gel. Agarose was boiled in TAE-buffer (Tris Acetate-EDTA buffer; Sigma), for DNA staining 0.1 µL/mL GelRed Nucleic Acid Stain (Biotium/VWR international) was added, and gels casted in appropriate chambers. PCR samples were diluted with 6x loading dye solution (Fermentas) and Gene Ruler 100 bp DNA Ladder Plus (Fermentas) was used to determine fragment size (150 bp). After electrophoresis at 120 V for 30 min, DNA separation and fragment sizes were analyzed with an UV transilluminator (Chemi Genius^2, Syngene, VWR

international). Before sequencing PCR products were isolated from agarose gels by QIAquick PCR Purification Kit (Qiagen). To analyze for pointmutation in murine DNA PCR samples were sequenced with the C1Inh_1_for primer using Operon/Eurofins sequencing service or sequencing service from the Karolinska Institute (KIgene, Stockholm, Sweden). A guanine at position +1 in the splice donor site of *Serping1* intron 2 classifies the analyzed animal as a WT, an adenine implicates a *Serping1*$^{-/-}$ mouse.

Antibodies against murine C1INH (α-C1INH) were generated by immunization of two rabbits using the peptide Cys-Asp^{23}-Pro^{24}-Glu^{25}-Ala^{26}-Thr^{27}-Ser^{28}-His^{29}-Ser^{30}-Thr^{31}-Gln^{32}-Asp^{33}-Pro^{34}-Leu^{35}-Glu^{36}–Amid. A Cys was N-terminal and an amid was C-terminal linked to the peptidsequence of murine C1INH (NP_033906). Peptidsynthesis and immunization was performed by BioGenes (Berlin, Germany). Before immunization pre-immune serum of each rabbit was collected for control. Immunizations, boosts, and collections of antisera were performed by standard protocols of BioGenes. After repeated boosting the animals were final bled to obtain sera. To control the presence of α-C1INH in rabbit sera plasma of human and mice (from WT and *Serping1*$^{-/-}$) was tested by Western blotting using 12% polyacrylamide gels. Western blotting detected α-C1INH Ab only in one rabbit (number 3312).

For immunoselection of α-C1INH Ab 2.4 mg of peptide dissolved in 10 mM sodium bicarbonate (pH 7.4; Roth) were coupled to an Affi-Gel 10 column (Bio-Rad) overnight. All steps were performed at 4°C. The column was washed five-times with water, two-times with wash buffer (2 M NaCl (Roth), 100 mM sodium acetate, pH 4 (Roth)), blocked with 1 M ethanolamine (pH 8) for 1 h, washed three-times with water and four-times with TBS (20 mM Tris, pH 7, 15 mM NaCl). Serum was diluted 1:1 with TBS and the mixture was incubated with the column overnight. The column was washed three-times with 10 mL of TBS, TBS-T and TBS, respectively. Thereafter, 1 mL fractions were eluted with elution buffer (200 mM glycine, pH 2, 150 mM NaCl) and 0.1 M Tris (pH 9) was immediately added. For regeneration the column was washed five-times

with PBS at RT. Eluted fractions were analyzed using SDS-page and Western blotting on 12% polyacrylamide gels. In fractions containing α-C1INH the buffer was exchanged with PBS with Econo-Pac 10DG columns from Bio-Rad according to the manufacturer's instructions.

α-C1INH Ab specifically detected C1INH in WT mouse plasma migrating with an apparent molecular weight of 94 kDa in SDS-PAGE. C1INH protein was not detectable by immunoblotting using Ab against C1INH in the plasma of *Serping1*$^{-/-}$ mice (Figure 5; (Oschatz et al., 2011).

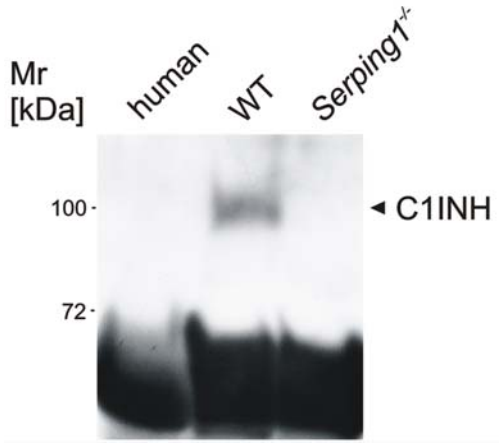

Figure 5. Analysis of murine C1INH expression. Human plasma (left lane), plasma from wild-type (WT) mouse (middle lane, 94 kDa) and *Serping1*$^{-/-}$ mouse plasma (right lane) was loaded. The unspecific signal of albumin (70 kDa) indicates equal lane loading (Oschatz et al., 2011).

4.2.10 Design of Hereditary angioedema type III patient survey

Thirty-eight hereditary angioedema patients of the Kantonsspital Luzern, Switzerland, with symptomatic deficiency in functional C1INH (<30% C1INH activity, 37 HAE type I, 1 patient with HAE type II, average age 40 years; 15 men 23 females of 11 families) were surveyed using the patient charts present at the outpatient clinic that was completed with a standardized questionnaire for which information was collected by personal or telephone interview. Diagnosis of allergic reactions was made based on clinical symptoms validated by experienced trained physicians. In patients with allergic reactions, allergens that trigger HAE attacks were identified using skin tests (prick test). Thirty-six patients suffered from abdominal edema and in 4 of them that was the sole symptom. 32 and 12 patients had edema in the skin and in the upper respiratory system, respectively. Ethical approval was granted from the ethical commission of the

Kanton Luzern and all patients had given informed consent. Design of Hereditary angioedema type III patient survey resulted from collaboration with W.A. Wuillemin and was published before in (Oschatz et al., 2011).

4.2.11 Generation of hereditary angioedema type III transgenic mice

1. Insertion of human FXII cDNA into TOPO TA vector.

cDNA from human FXII (rzpd, Berlin, Germany; MIM ID: 610619) was amplified using an Advantage-HF 2 PCR Kit (TaKaRa/Clontech, Saint Germain en Laye, France; High-Fidelity (HF)). PCR reaction was performed in 50 µL reaction volume containing 5 µL HF buffer, 10 µM each of forward and reverse primer (forward primer *Hin*dIII: 5´-GTATAAGCTTGATCTGGACTCCTGGATAGG-3´; reverse primer *Xba*I: 5´-GTAATCTAGAGGGGAATGGGACACAATCTT-3´; Eurofins), 10 mM dNTP mix, HF Polymerase and 100 ng cDNA of human FXII. The PCR program was: preincubation at 94°C for 2 min, 30 cycles of 94°C for 30 sec and 68°C for 2 min. Final step was 68°C for 3 min. Amplified FXII of 1.8 kbp were separated by gelelectrophoresis, and extracted from gel by QIAquick Gel Extaction Kit (QIAGEN). FXII was inserted into a TOPO TA vector (Invitrogen, Karlsruhe, Germany) using the TOPO TA cloning Kit (Invitrogen) following manufacturer´s instructions to generate the TOPO TA_FXII vector.

2. Generation of FXII pointmutation in TOPO TA_FXII vector

1032C→A pointmutation was generated in FXII in the TOPO TA_FXII vector by site-directed mutagenesis using QuikChange Multi Site-directed Mutagenesis Kit (Stratagene, Amsterdam, the Netherlands). The mutagenic FXII-Thr328Lys primer (5`-CCGAAGCCTCAGCCCA**A**GACCCGGACCCCGCCTCAG-3´, sequence variation with respect to the FXII cDNA is in bolt; TIB MOLBIOL, Berlin, Germany) was employed to exchange C 1032 with A. PCR reaction was performed in 25 µL reaction volume containing 2.5 µL QuikChange Multi reaction buffer, 0.5 µL QuikSolution, 100 ng TOPO

TA_FXII vector, 0.64 µM mutagenic FXII-Thr328Lys primer, 10 mM dNTP mix and 2.5 U/µL QuikChange Multi enzyme blend. For PCR preincubation at 95°C for 1 min, 30 cycles of denaturation step at 95°C for 1 min, annealing step at 55°C for 1 min and elongation step at 65°C for 11 min were performed. Resulting TOPO TA vector with a 1032C→A mutated human FXII was named TOPO TA_FXIIThr328Lys. Following the manufacturer´s instructions (Stratagene) the vector was demethylated with 15 U *Dnp*I for 2 h at 37°C.

3. Subcloning of mutated FXII from TOPO TA_FXIIThr328Lys vector into pcDNA3 vector.

Restriction digestion of the TOPO TA_FXIIThr328Lys vector and pcDNA3 vector (Invitrogen) were performed in 50 µL reaction volumes, each, containing 5 µg DNA, 5 µL restriction buffer 2, 5 µL BSA and 40 U of restriction enzymes *Hin*dIII (5`) and *Xba*I (3´) (NEW ENGLAND BioLabs, Frankfurt, Germany). Reactions were incubated at 37°C for 3 h. After restriction digestion fragment size were controlled with agarose gelelectrophoresis. Mutated FXII (1.8 kbp) and cleaved pcDNA3 vector (5.4 kbp) were extracted from gel using QIAquick Gel Extaction Kit (QIAGEN). Ligation of 50 ng mutated FXII fragment with 50 ng pcDNA3 vector was set up in a 20 µL reaction volume using 2 µL T4 DNA ligase buffer and 400 U T4 DNA ligase (NEW ENGLAND BioLabs). Ligase reaction was incubated 5 min at RT resulting in pcDNA3_FXIIThr328Lys vector. Ligase reaction was controlled by restriction digestion. pcDNA3_FXIIThr328Lys vector was transformed into competent *E. coli* (One shot TOP10 Competent Cells; Invitrogen) according to manufacturer´s instructions. Transformed *E.coli* were grown over night at 37°C on agar plates (Lysogeny broth medium (1 % (w/v) tryptone (Roth), 0.5 % (w/v) bacto yeast extract (Roth), 1 % (w/v) NaCl, pH 7.0) and 1.5 % (w/v) agar (Roth)) in the presence of 100 µg/mL ampicillin (Sigma). pcDNA3_FXIIThr328Lys plasmid DNA was isolated from exponentially

growing bacterial culture (at 37°C shaking at 200 rpm; OD_{600} = 0.6) according to manufacturer´s instructions (QIAfilter Kit for plasmid purification, Qiagen).

4. Subcloning of mutated FXII from pcDNA3_FXIIThr328Lys into pTRE-Tight vector.

Restriction digestion of both, pcDNA3_FXIIThr328Lys plasmid and pTRE-Tight vector (TaKaRa/Clontech) was performed for 3 h at 37°C in a 100 µL reaction volume containing 10 µL BSA, 10 µL buffer 2, 13 µg pcDNA3_FXIIThr328Lys vector, 40 U *Hin*dIII (5`) and 40 U *Eco*RV (3`) (NEW ENGLAND BioLabs). After restriction digestion fragment size was controlled with agarose gelelectrophoresis and fragments were extracted from the gel. FXIIThr328Lys DNA fragment (1.8 kbp) was ligated with pTRE-Tight vector (2.6 kbp) to pTRE-Tight_FXIIThr328Lys. 20 µL ligation volume contained 50 ng pTRE-Tight plasmid vector, 104 ng FXIIThr328Lys DNA fragment, 2 µL Ligase buffer and 400 U T4 DNA Ligase. The reaction batch was mixed, incubated for 30 min at 16°C and used for transformation. Map of pTRE-Tight_FXIIThr328Lys vector is shown in Figure 6. pTRE-Tight_FXIIThr328Lys plasmid DNA was isolated from bacteria culture. Size of FXII, sequence of human FXII according to MIM ID: 610619 and 1032C→A pointmutation were confirmed by control restriction digestion and DNA sequence analyses using Operon/Eurofins sequencing service (Eurofins).

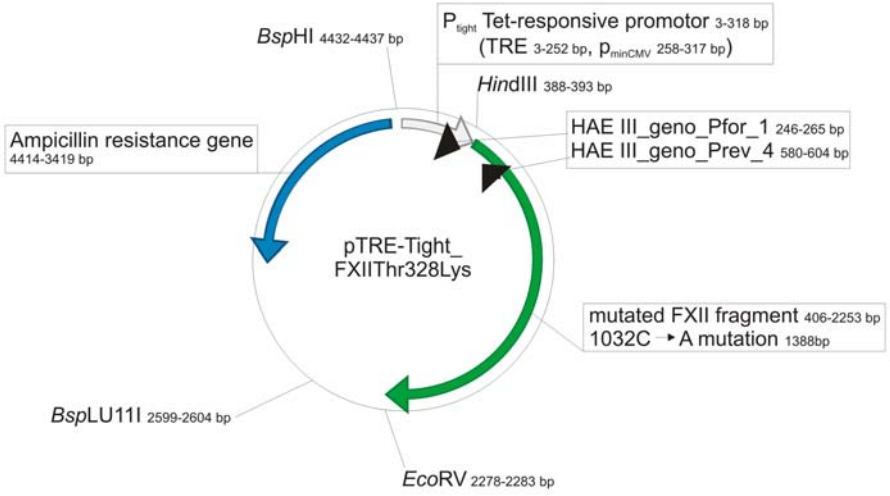

Figure 6. Map of pTRE-Tight_FXIIThr328Lys vector. The vector contains a Tet-responsive promoter, the 1032C→A mutated human FXII and an ampicillin resistence gene. Location of forward and reverse primer (HAE III_geno_Pfor_1, HAE III_geno_Prev_4) for genotyping of HAE III mice is indicated. Description of containing features is given in boxes. Recognition sites of restriction enzymes are indicated without boxes. Positions in the vector are given in numbers of bp.

5. Generation of a HAE III mouse

To generate a HAE III mouse pTRE-Tight_FXIIThr328Lys vector was isolated from *E. coli* bacteria cultures (QIAfIter Kit for plasmid purification, Qiagen). Restriction digestion, agarose gelelectrophoresis and sequencing analysis (Eurofins) confirmed the right size of 1.8 kbp, sequence and the 1032C→A pointmutation of the human FXII, before the vector was send to the Institute of Experimental Pathology, Münster, Germany. HAE III mouse was generated via oocytes injection of pTRE-Tight_FXIIThr328Lys plasmid after linearization with *Bsp*HI (5´) and *Bsp*LU11I (3`) (Figure 6). 31 pups were born. For genotyping of HAE III mice the genomic DNA was isolated from mice tails with a DNeasy blood & tissue Kit (Qiagen). For PCR a Top Taq DNA Polymerase Kit (Qiagen) was used. PCR reactions were performed in 25 µL reaction volume containing 2.5 µL Top Taq buffer, 5 µL Q-solution, 0.8 µM each of forward and reverse primer (forward primer HAE III_geno_Pfor_1: 5´-CGTATGTCGAGGTAGGCGTG-3´; reverse primer HAE III_geno_Prev_4: 5´-CACAAATGTACCCACAAGGGCCGGC-3´; Eurofins; Figure 6), 0.4 mM dNTP mix, 5 U Top Taq DNA Polymerase and 50-100 ng genomic DNA. The cycling condition were: preincubation at 94°C for 3 min, 30 cycles of denaturation step at 94°C for 30 sec, annealing step at 58°C for 30 sec and elongation step at 72°C for 1 min. Final elongation step was at 72°C for 10 min. Mutated FXII fragments of 359 bp of transgenic mice were separated by gelelectrophoresis on 1 % agarose gel. 3/31 (10%) of the pups carried the transgene.

4.2.12 Data analysis

All data were represented as means ± SD. Statistical analyses were performed using the one-way ANOVA, followed by Dunnett´s post test. P-values < 0.05 were considered statistically significant (Oschatz et al., 2011).

5 RESULTS

5.1 Mast cell-heparin activates the contact system in plasma

High molecular heparin (500-750 kDa) was isolated from rat peritoneal MC and the potency of the glycosaminoglycans for BK generation was analyzed. Human plasma was incubated with increasing amounts of MC-heparin and analyzed for HK processing and BK formation by Western blotting and ELISA, respectively. Antibodies against the BK sequence in HK revealed that ≥4 µg/mL MC-heparin initiated complete plasma HK cleavage (Figure 7A, upper panel). Consistently, BK was high (>780 ng/mL) in these samples, but low in those treated with buffer (37 ± 22 ng/mL) or with ≤2 µg/mL MC-heparin (<35 ng/mL), which was not sufficient to initiate HK processing (Figure 7A, bar graph). As a control, MC-derived glycosaminoglycans were incubated with heparinase (Hase) before adding them to plasma. The enzyme specifically digests heparin. Hase-treatment largely abolished BK formation, triggered by activated MC-released material. HK conversion started at >30-fold higher concentrations of the digested (≥128 µg/mL, Figure 7B) *vs.* non-treated glycosaminoglycans, indicating that heparin accounts for most of the BK-forming activity in MC-released material. Heparin induces the reciprocal activation of FXII and PK in ice-cold plasma (Hojima et al., 1984). Consistent with this observation, MC-heparin failed to initiate HK cleavage and BK production in FXII or PK deficient plasma at physiological temperature (Figure 7C). In contrast, deficiency of the FXII substrate in the intrinsic coagulation pathway, FXI, did not interfere with HK processing (Figure 7C, lane 2). MC-heparin added to normal plasma induced the contact system cascade with sequential proteolytic activation of FXII, PK and HK, but not of LK (a kininogen gene splice variant that does not bind PK) or FXI (Figure 7D). Efficient contact system activation depends on negative charge density and the size of the FXII-binding surface (Muller and Renne, 2008).[1]

[1] The text of the whole page is taken from the publication Oschatz et al., 2011.

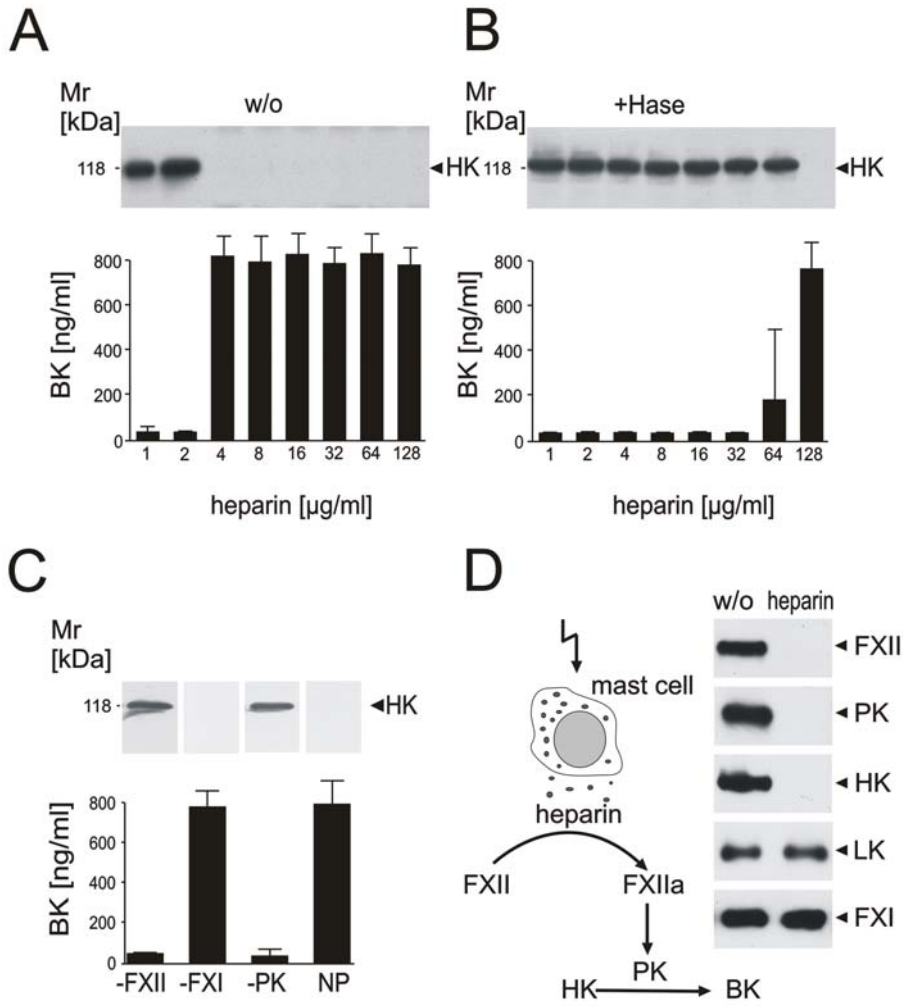

Figure 7. MC-derived heparin initiates contact system-mediated BK formation. Human plasma was incubated for 30 min at 37°C with MC-derived glycosaminoglycans and analyzed for single-chain HK by Western blotting, and for BK formation by ELISA (means ± SD, n=8). Glycosaminoglycans at ≥4 µg/mL initiated plasma HK cleavage and BK formation (A). MC-derived glycosaminoglycans were incubated with heparinase (Hase) prior to addition of plasma (B). Plasma deficient in FXII, FXI, or PK and normal plasma (NP) was incubated with 20 µg/mL MC-heparin for 30 min and analyzed for HK cleavage and BK liberation (C). *Left*: Schematic drawing of MC-heparin triggered reaction cascade, which forms BK by FXII/PK-mediated HK processing. *Right*: Buffer- (w/o) and heparin-treated (heparin) plasma was probed for FXII, PK, single-chain HK, LK, and FXI zymogen, respectively (D) (Oschatz et al., 2011). These data resulted from a collaboration with T. Renné.

MC-heparin and heparan sulfate were compared for their ability to release BK from HK. Both polysaccharides have similar backbones consisting of alternating iduronic acids and glucosamines, but differ in charge (heparin has an average of 2.7 sulfate per groups typical disaccharide unit, whereas heparan sulfate has an average of 1.0;[2]

[2] This text section is taken from the publication Oschatz et al., 2011.

(Capila and Linhardt, 2002)). Human plasma was incubated with increasing amounts of MC-heparin, heparan sulfate and the synthetic polysaccharide dextran sulfate potent FXII activator; (Johne et al., 2006), respectively (Figures 8A - 8C). MC-heparin and dextran sulfate at ≥10 µg/mL each, triggered complete conversion of plasma HK. In contrast, heparan sulfate up to 100 µg/mL did not induce HK cleavage and high[3]

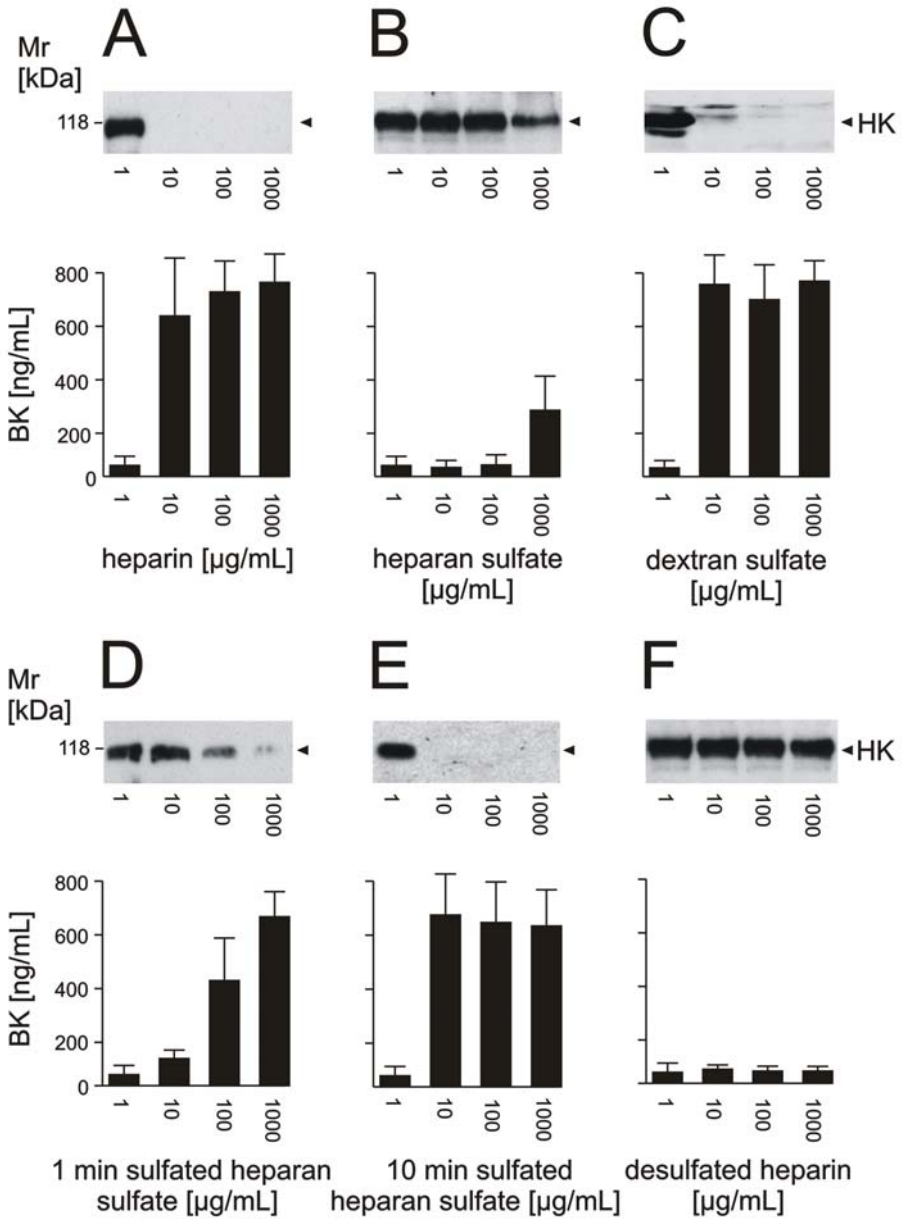

Figure 8. MC-heparin-induced BK formation is dependent on the negative charge density of the polyanion. Human plasma samples were supplemented with 1 to 1000 µg/mL heparin (A), heparan sulfate (B), dextran sulfate (C), heparan sulfate sulfated for 1 min (D) or 10 min (E) and desulfated heparin (F), respectively, incubated for 30 min at 37°C, and analyzed for HK cleavage and BK formation by Western blotting and ELISA, respectively. Means ± SD, n=8 are plotted (Oschatz et al., 2011). These data resulted from a collaboration with T. Renné and B. Lecher.

[3] This text section is taken from the publication Oschatz et al., 2011.

concentrations (1000 µg/mL) of the glycosaminoglycan only induced minor HK cleavage (36% of total plasma HK, corresponding to 260 ng/mL BK). To demonstrate that negative charge density, rather than a defined structure of the polysaccharide is responsible for the enhanced BK-generating potency of heparin as compared to heparan sulfate, heparan sulfate was sulfated with sulfuric acid/carbodiimide. This method does not cause depolymerization of the polysaccharide. Hypersulfation increased the BK-forming activity of heparan sulfate (Figure 8D). After 10 min sulfation, the activity of treated heparan sulfate was similar to heparin (compare Figure 7E *vs.* 8A). In contrast, chemical desulfation of heparin with N,O-bis(trimethylsilyl)acetamide largely abolished its BK-forming activity (Figure 8F). Together, the data show that MC-heparin initiates BK formation in plasma via activation of the contact system.[4]

5.2 Mast cell-heparin competes with high molecular weight kininogen for binding to cells

BK formation is tightly regulated and HK binding to the cell surface heparan sulfate-type proteoglycans protects the BK-precursor from proteolysis (Renne et al., 2005b). Because both heparin and heparan sulfate bind to the cell-binding site in HK (Leeb-Lundberg et al., 2005) MC-heparin might detach cell-bound HK from heparan sulfates as a prerequisite for efficient BK formation. To test this hypothesis, EA.hy926 endothelial cells were incubated with 60 nM radiolabeled, uncleaved HK, removed unbound HK was removed and HK deficient plasma supplemented with MC-heparin were added (0.05-200 µg/mL). This experimental design analyzes cell-bound HK in the absence of HK in the fluid phase. Following incubation, cell-bound HK, HK cleavage, and BK formation were monitored. Heparin dose-dependently reduced HK-cell binding; at ≥4 µg/mL polysaccharide, cell-bound HK was reduced to <20% of levels measured[5]

[4] This text section is taken from the publication Oschatz et al., 2011.
[5] This text section is taken from the publication Oschatz et al., 2011.

in the absence of heparin (set to 100%; Figure 9A). HK detachment from cells was associated with HK cleavage (Figure 9B) and BK formation (Figure 9C).[6]

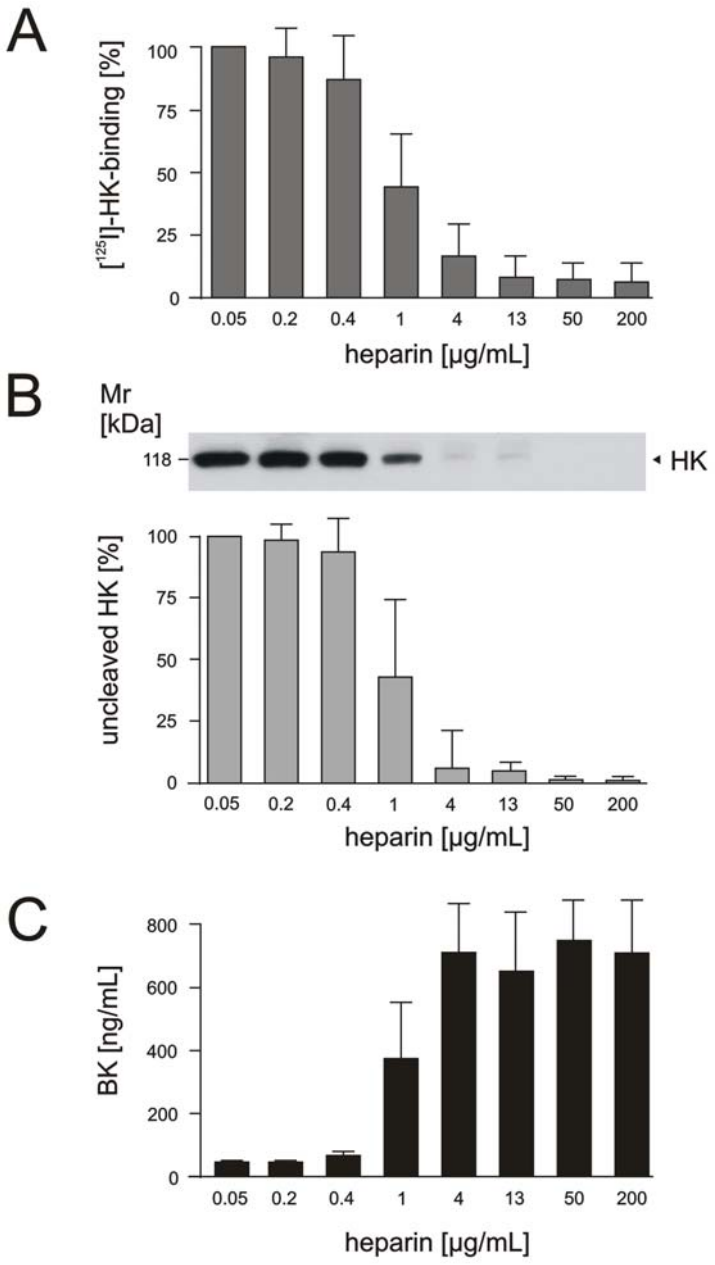

Figure 9. MC-heparin detaches HK from endothelial cells and induces BK release. Uncleaved radiolabeled HK (60 nM) bound to EA.hy926 cells was incubated with 0.05 - 200 µg/mL heparin in HK deficient plasma. Following incubation for 30 min at 37°C, the samples were analyzed for HK cell binding, HK cleavage, and BK formation. The cellular [^{125}I]-HK binding capacity was monitored by a γ-counter and normalized for HK binding in the absence of heparin (set at 100%) (A). Cleavage of HK was analyzed by Western blotting using antibodies against the BK sequence (MBK3). The relative levels of uncleaved HK, determined from Western blot signal intensities are given below (untreated control set at 100%) (B). ELISA measured BK in cell supernatants (C). Means ± SD of 5 independent experiments are shown (Oschatz et al., 2011). These data resulted from collaboration with T. Renné.

[6] These two lines are taken from the publication Oschatz et al., 2011.

5.3 Mast cell-heparin activates the contact system in rodents.

To test MC-heparin for triggering BK generation in rodents, the polysaccharide was infused into the femoral artery of rats and BK formation was analyzed by ELISA. Heparin injection greatly increased plasma BK (224 ± 93 ng/mL) over buffer-treated animals (23 ± 16 ng/mL). BK generation was associated with leukocyte adhesion to vessel walls, as shown in limb cross-sections from animals that were sacrificed 30 min after challenge (Figure 10A). Leukocyte-endothelial cell adhesion was restricted to femoral artery vessel walls downstream of the injection site and to the ipsilateral *vena femoralis*. At some sites, leukocytes had penetrated the endothelial border and invaded into subendothelial tissues (Figure 10B). To confirm that BK mediates MC-heparin-triggered leukocyte adhesion, we treated rats with the B2R antagonist Icatibant (Wirth et al., 1991) prior to heparin infusion. Icatibant did not alter heparin-induced BK plasma levels (181 ± 72 ng/mL) but almost completely blunted leukocyte adhesion to vessel walls (Figures 10C and 10D). In addition to being a chemoattractant for neutrophils, BK is a potent vasodilator (Leeb-Lundberg et al., 2005). Intraarterial MC-heparin infusion induced a rapid (within 45 sec), pronounced (≈160 to 50 mmHg) and reversible drop in blood pressure. Blood pressure recovered close to baseline levels within 30 min. Heparin-triggered hypotonic reaction was almost completely blocked by Icatibant (Figure 10E). In contrast to intraarterial application, subcutaneous or intravenous injection of MC-heparin up to 25 mg/kg bw did not change blood pressure nor trigger leukocyte adhesion (not depicted).

To evaluate the role of MC-heparin-driven BK formation for allergic reactions we challenged wild-type, FXII deficient (*F12*$^{-/-}$) and B2R deficient (*Bdkrb2*$^{-/-}$) mice that are defective in BK generation (Pauer et al., 2004) or BK signaling (Borkowski et al., 1995), respectively, in a model of passive systemic anaphylaxis. We injected mice intravenously with α-DNP IgE and challenged them 24 h later with an intravenous injection of DNP-HSA. WT mice responded with a rapid[7] and transient drop in systemic

[7] The text of the whole page is taken from the publication Oschatz et al., 2011.

arterial blood pressure of 57 ± 24 mmHg, whereas allergen-induced hypotonic response was decreased in *F12*$^{-/-}$ (23 ± 10 mmHg) and *Bdkrb2*$^{-/-}$ (27 ± 13 mmHg) mice.[8]

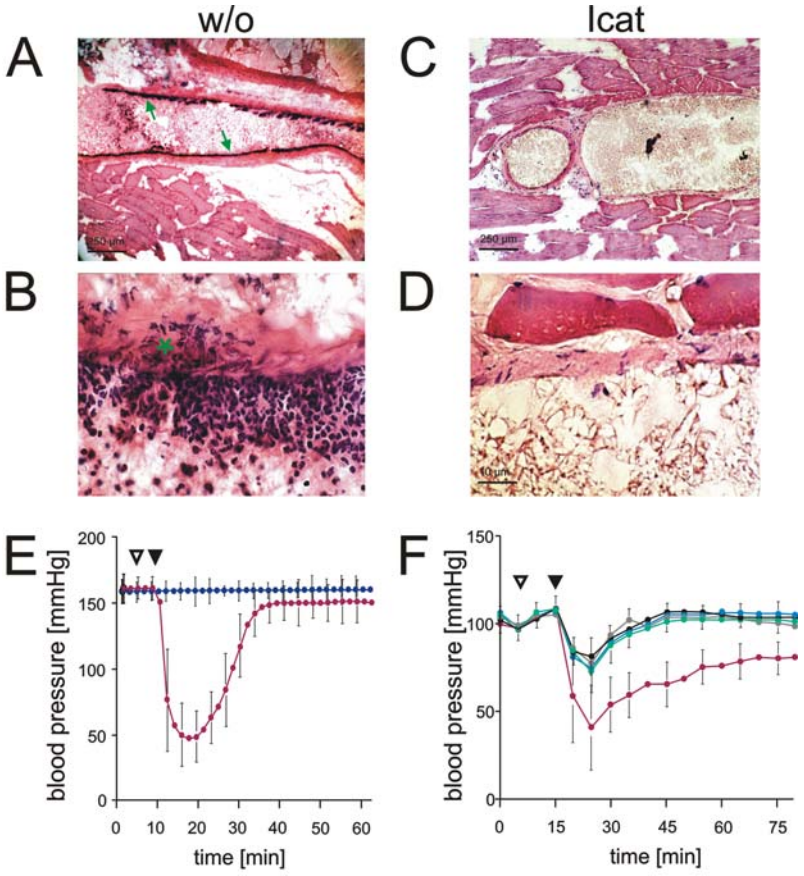

Figure 10. MC-heparin induces BK-driven leukocytes infiltration and hypotension in rodents. Heparin was infused into the left femoral artery of untreated (w/o) or Icatibant (Icat) treated rats and the animals were sacrificed 30 min after challenge. Hematoxylin and eosin stained cross- sections of limb vessels dorsal to the heparin infusion site are shown. Leukocytes adhere to vessel walls (green arrows) in untreated animals (A) and transmigrate through the endothelial barrier (asterisk,higher magnification) (B).Corresponding sections of Icat-treated animals are shown in (C) and at higher magnification in (D). Bar is 250 µm in A and C, and 10 µm in B and D. Representative histology of n=7 rats in each group is shown. Heparin was injected into the aorta of rats and the mean arterial blood pressure was measured in the left carotid artery in untreated- (red line) or Icat-pretreated rats (blue line). The times of Icat and heparin administration are indicated by white and black triangles, respectively. Means ± SD, n=7 rats (E). Arterial blood pressure during IgE/Ag-induced systemic anaphylaxis in WT (red), *F12*$^{-/-}$ (black) and *Bdkrb2*$^{-/-}$ (green) mice or WT mice pretreated with PCK (gray) or Icat (blue). Infusion of Icat or PCK and DNP-HSA is symbolized by white and black triangles, respectively. Means ± SD of 10 mice per group are displayed (F). Western blot analyses of plasma using antibodies against HK (non-processed single-chain HK, upper row), the BK sequence in HK (middle row), and FXI (lower row) in plasma of IgE/Ag- (lane 1), IgE/buffer-challenged (lane 2), and untreated mice (w/o; lane 3) (G) (Oschatz et al., 2011). The data of 11A-F resulted from collaboration with B. Lecher.

[8] This text is taken from the publication Oschatz et al., 2011.

HK cleavage and BK release in plasma of IgE/Ag-challenged mice were analyzed. Ten min after antigen infusion BK was released from circulating HK in anaphylactic mice (Figure 10G).

WT mice pretreated with either FXII-inhibitor PCK (Kleinschnitz et al., 2006) or Icatibant were protected from IgE/antigen (Ag)-mediated drop in blood pressure (25 ± 10 and 29 ± 12 mmHg; Figure 10F). The aPTT is a commonly used diagnostic coagulation test to measure heparin plasma activity. IgE/Ag challenge largely prolonged the aPTT[9] compared to IgE/buffer-infused control mice. Indeed, plasma samples from anaphylactic mice (n=15) did not clot at all (clotting times >180 seconds *vs.* 46 sec in buffer treated animals) indicating considerable heparin release (exceeding the amount used in anticoagulation therapy). A 1:10 dilution of plasma of anaphylactic mice with plasma of untreated mice led to a measurable prolongation of the aPTT (73.9 ± 24.2 sec, n=10) corresponding to 4.7 ± 1.7 µg/mL heparin, 10 min after IgE/Ag challenge in undiluted plasma of IgE/Ag treated mice. This heparin concentration is sufficient to induce activation of FXII and PK, cleavage of HK, and BK formation (starting at >2 µg/mL, Figure 7A). At 5 min after challenge, the systemic heparin concentration was 0.8 µg/mL for C48/80 challenge and 2.1 µg/mL for IgE/Ag challenge, respectively. Addition of the heparin antidote protamine (Goto et al., 1984) reduced the aPTT (51.2 ± 7 sec, n=5) close to levels of untreated control mice (46 ± 2 sec, n=15). Similarly, incubation of plasma of IgE/Ag treated mice with heparinase (the enzyme that blocked heparin-driven BK formation, Figure 7B) reversed the IgE/Ag-induced aPTT prolongation (57.3 ± 20.6 sec, n=5). As protamine- and heparinase reversed the prolonged aPTT during anaphylaxis, this shows that a considerable amount of heparin is released during anaphylaxis. Consistent with IgE/Ag challenge in mice, infusion of C48/80 significantly prolonged the aPTT (>3 fold over control). Since heparin is rapidly cleared from the circulation with a plasma half-life of approximately 60 min (Bjornsson and Levy, 1979), the coagulation assays probably underestimate the amount of

[9] These four lines are taken from the publication Oschatz et al., 2011.

released heparin. Additionally, the local heparin concentration at the MC surface may largely exceed the measured systemic concentration of the polysaccharide.

Cumulatively, the data show that MC derived-heparin initiates BK formation via the FXII-stimulated contact system and that this pathway contributes to MC-driven hypotonic reactions in systemic anaphylaxis *in vivo*.[10] The data are consistent with heparin release and aPTT prolongation during anaphylactic reactions in patients (Mazzi et al., 1994; Lombardini et al., 2009).

5.4 Mast cell-heparin increases microvascular permeability in a bradykinin-dependent manner in mice

A hallmark of BK-mediated effects is increased vascular permeability (Han et al., 2002). To analyze contact system-mediated leakage in genetically altered mice, an intravital confocal scanning microscopy technique was established to visualize microvascular fluid efflux. Macromolecular FITC-dextran was intravenously injected as a tracer for paracellular extravasated plasma proteins. A ventral skin window was incised, skin was inverted and analyzed under the microscope. Fluorescence tracer showed blood flow in microvessels during the experiment. No basal tracer extravasation was detectable for 3 min prior to stimulation, indicating intact vascular barriers. Topical application of MC-heparin (time point 0 min) provoked leakage from capillaries in WT mice. First leaky spots appeared within 10 min and leakage was maximal after 20 min (tracer fluorescence intensity 67.1 ± 15.2-fold of initial t=0 min signal; Figure 11, column 1). To exclude that the increase in vascular permeability is mediated by a contaminant rather than by heparin, the polysaccharide was digested with Hase. Hase-treatment abolished the activity of the polysaccharide to increase vascular leakage in WT mice (1.6 ± 0.9-fold at 20 min; column 2). To exclude a[11]

[10] These three lines are taken from the publication Oschatz et al., 2011.
[11] This text section is taken from the publication Oschatz et al., 2011.

contribution of released endogenous MC-heparin MC deficient mice (*Kit*$^{W\text{-}sh/W\text{-}sh}$) were tested. Exogenous MC-heparin-triggered leakage in MC deficient mice (64.2 ± 4.2; column 5) to similar levels as observed in WT animals. Because FXII-independent mechanisms for PK activation exist (Schmaier, 2008), we analyzed heparin-triggered leakage in *F12*$^{-/-}$ mice that are defective in contact system-driven BK formation (Pauer et al., 2004). Intravital microscopy showed that *F12*$^{-/-}$ animals were resistant to MC-heparin-stimulated alterations in vascular permeability (1.6 ± 0.7, 20 min; column 3). To confirm that MC-heparin induces vascular leakage by releasing BK, *Bdkrb2*$^{-/-}$ mice were employed that are protected from BK-driven edema (Han et al., 2002). *Bdkrb2*$^{-/-}$ animals were mostly resistant to heparin-induced increase in permeability (1.7 ± 1.4, 20 min; column 4). Histamine triggered leakage in *F12*$^{-/-}$ and *Bdkrb2*$^{-/-}$ mice (63.3 ± 8.7 and 65.8 ± 11.8; column 7), indicating that these animals are susceptible for plasma contact system-independent edema formation.

HAE is characterized by recurrent swelling attacks. The underlying disease mechanism is a deficiency in functional C1INH (Zuraw, 2008). In patients with inherited C1INH deficiency, poorly defined stimuli trigger contact system-driven excessive BK formation that increases vascular leakage (Davis, 2008). To generate an animal model for HAE, the *Serping1* gene in mice was inactivated, as described in material and methods. Since MC contribute to allergic angioedema (Greenhawt and Akin, 2007), MC-heparin was investigated as a potential trigger for vascular leakage in C1INH deficient (*Serping1*$^{-/-}$) mice. Intravital microscopy showed that MC-heparin provoked excessive vascular leakage in *Serping1*$^{-/-}$ *vs.* WT mice (compare column 6 *vs*. 1). Leakage began earlier (first leaky spots at 4.5 min *vs.* 10 min), was longer-lasting (>50 min) and was greatly increased in *Serping1*$^{-/-}$ over WT mice (113 ± 17.2 *vs.* 67.1 ± 15.2, t=20 min).[12]

[12] The text of the whole page is taken from the publication Oschatz et al., 2011.

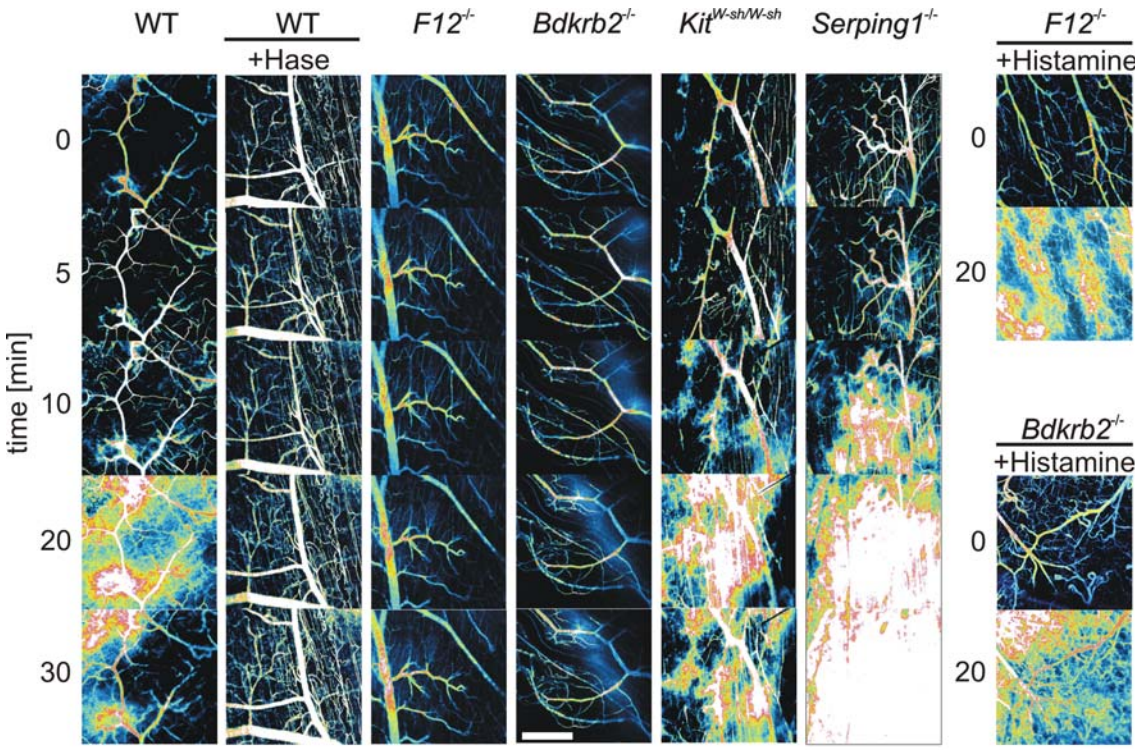

Figure 11. MC-heparin increases permeability of skin microvessels. Extravasation of FITC-dextran tracer from murine dorsal skin microvessels was recorded by intravital laser scanning fluorescence microscopy in real time. Heparin was topically applied to the skin of WT, *F12*$^{-/-}$, *Bdkrb2*$^{-/-}$, *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Serping1*$^{-/-}$ mice (columns 1, 3-6). For control, heparinase (Hase) treated heparin was applied to the skin of WT animals (column 2) and histamine to *F12*$^{-/-}$ and *Bdkrb2*$^{-/-}$ mice (column 7). Laser scanning images were taken at 5, 10, 20 and 30 min after stimulation at time-point 0 min and are shown in false colors. White represents the highest tracer intensity. The scale bar represents 500 µm. Confocal images were recorded with a 10x objective and processed with software EZ-C1, version 2.10 for Nikon. A typical experiment of a series of n=10 mice per genotype is shown (Oschatz et al., 2011).

5.5 Mast cell-heparin induces contact system-driven edema in mice

The relative importance of heparin-driven BK formation for MC-mediated leakage in mice was analyzed using the Miles edema model (Miles and Miles, 1952). Intradermally injected stimuli triggered leakage in dermal vessels that was visualized by Evans blue tracer. Extravasated tracer was extracted from the tissue, quantified by fluorescence emission and plotted relative to the NaCl-induced signal in WT mice (set to 1.0). Basal vascular permeability, assessed in NaCl injected skin, was low (≤1.2) in[13]

[13] The last four lines are taken from the publication Oschatz et al., 2011.

all tested mice. BK-stimulated leakage was similar in WT (Figure 12A) and *F12*$^{-/-}$ (Figure 12B) mice (4.2 ± 0.9 *vs*. 4.1 ± 0.7; Figure 12F) whereas *Bdkrb2*$^{-/-}$ animals were resistant to BK challenge (1.2 ± 0.5; Figures 12C and 12F). Heparin injection induced edema in WT animals (3.3 ± 0.8), whereas *F12*$^{-/-}$ and *Bdkrb2*$^{-/-}$ mice were almost completely resistant to heparin-induced leakage and edema was not significantly different from NaCl controls (1.3 ± 0.4 *vs*. 1.1 ± 0.3 and 1.1 ± 0.3 *vs*. 1.1 ± 0.2, Figure 12F).[14]

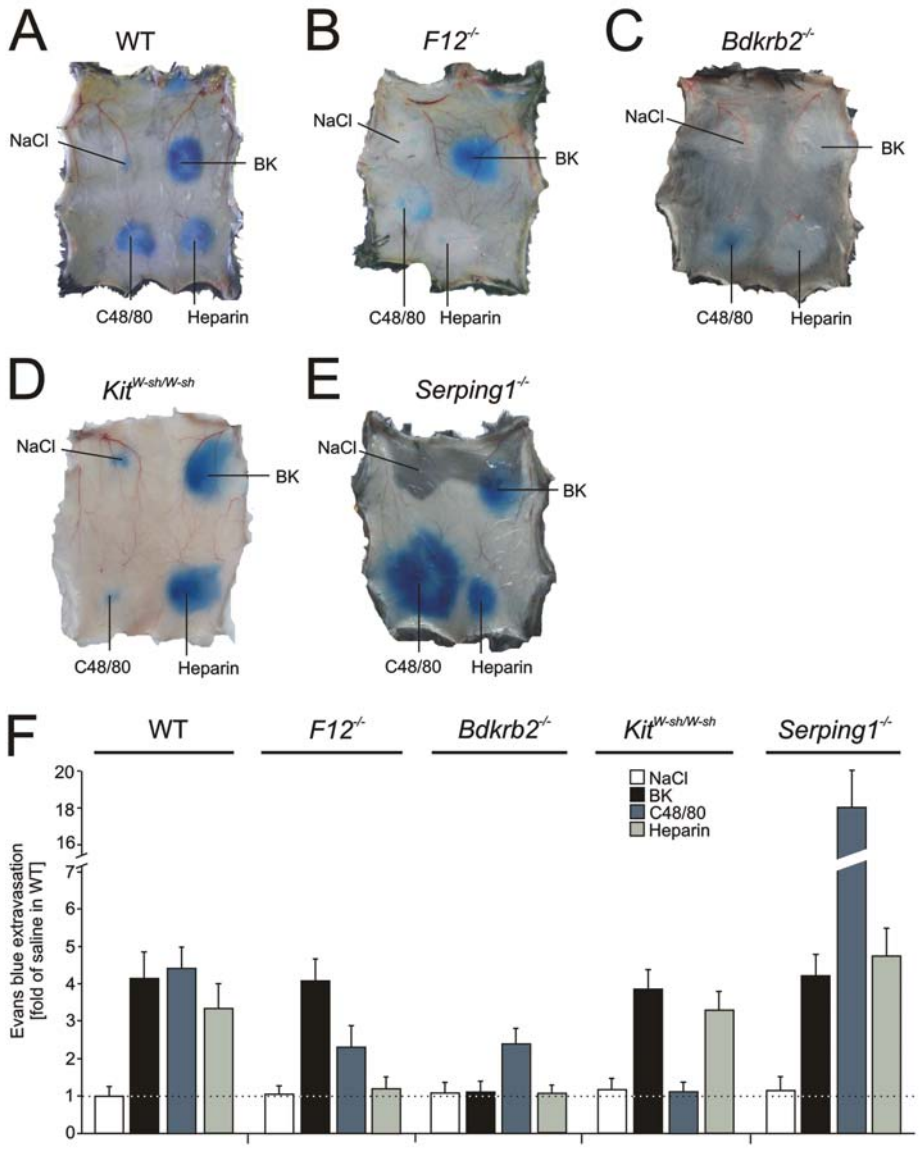

Figure 12. MC-heparin initiates edema in a BK-dependent manner. Evans blue was infused intravenously as a tracer into WT (A), *F12*$^{-/-}$ (B), *Bdkrb2*$^{-/-}$ (C), *Kit*$^{W\text{-}sh/W\text{-}sh}$ (D) and *Serping1*$^{-/-}$ (E) mice. Skin edema was induced by intradermal injections with BK, C48/80 and heparin or NaCl. Leakage was analyzed after 30 min. Spots with extravasated tracer were entirely excised, dye was extracted, and quantified. Tracer extravasation is plotted relative to leakage in WT mice stimulated with NaCl. Columns show means ± SD, n=10 per group (F) (Oschatz et al., 2011).

[14] This text section is taken from the publication Oschatz et al., 2011.

Since congenital deficiency in FXII and B2R provides protection from edema, pharmacological inhibition of BK formation was tested and signaling for interference with heparin-driven leakage. Infusion of PCK or Icatibant prior to challenge largely reduced heparin-induced edema in WT mice as compared to untreated animals (1.7 ± 0.5 and 1.5 ± 0.5 *vs*. 3.0 ± 0.6; Figure 13).

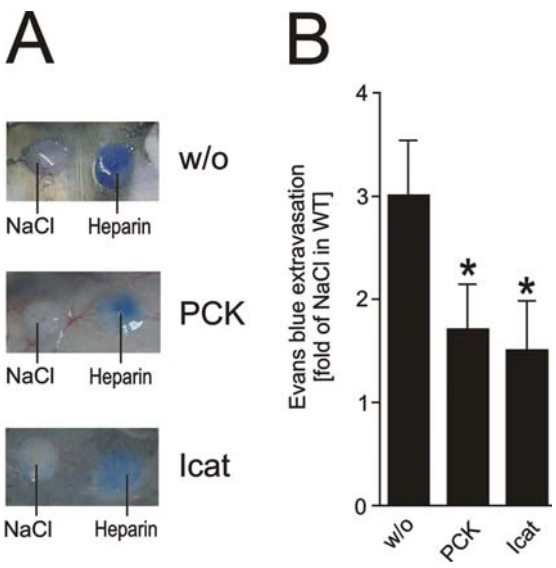

Figure 13. Pharmacological targeting of the contact activation system interferes with MC-heparin-induced edema in WT mice. Mice were intravenously injected with NaCl (w/o), FXIIa-inhibitor PCK (8 µg/g bw), or B2R antagonist Icatibant (Icat, 175 µg/kg bw), respectively. Five min later, they received an intradermal injection of NaCl (left) or heparin (right) (A). Extravasated Evans blue tracer was determined 30 min after challenge and is shown relative to levels of NaCl-induced leakage in untreated WT animals. Columns give means ± SD of n=10 animals per group (B) (Oschatz et al., 2011).

Tracer measurements are consistent with the intravital microscopy data (Figure 13). Cumulatively, the data show that heparin is an activator of the contact system and that the polysaccharide initiates BK-driven edema *in vivo*. To determine the importance of heparin-initiated BK formation for activated MC-driven skin edema, animals were challenged with compound 48/80 (C48/80), a degranulator of MC and eosinophiles (Sher and Wadee, 1981). MC deficient mice were largely resistant to C48/80 stimulation and leakage was low as in NaCl controls (1.1 ± 0.4 *vs*. 1.2 ± 0.3; Figures 12D and 12F). In *F12*$^{-/-}$ animals C48/80-stimulated leakage was reduced to 2.3 ± 0.6-fold that is 52% of WT mice level (4.4 ± 0.6; Figures 12B and 12F). *Bdkrb2*$^{-/-}$ mice confirmed that FXII-triggered BK formation, rather than other FXII-initiated pathways, is important for MC-induced edema. In these animals C48/80-provoked leakage was[15]

[15] The whole text of this page is taken from the publication Oschatz et al., 2011.

reduced to 55% (2.4 ± 0.5-fold; Figures 12C and 12F) of WT mice levels. The data indicate that heparin-induced BK formation accounts for about half of the edema-forming capacity of activated MC. To test heparin for triggering edema in HAE, the *Serping1*$^{-/-}$ mice were employed (Figures 12E and 12F). NaCl-induced leakage was 1.2 ± 0.4-fold of WT mice, indicating that basal vascular permeability in *Serping1*$^{-/-}$ mice was not significantly different from that of WT animals ($p > 0.05$). In contrast, application of heparin or C48/80 triggered excessive edema that exceeded levels in WT mice by >45% and >60% (4.8 ± 0.7 and 18.1 ± 2.0), respectively, whereas BK-driven edema was not significantly different from levels in WT animals (4.3 ± 0.6; $p > 0.05$).[16]

To determine downstream BK signaling the role of its downstream target VASP was investigated and VASP deficient (*VASP*$^{-/-}$) mice were employed. In the Miles edema model WT and *VASP*$^{-/-}$ mice were intradermally injected with BK and NaCl (control). Evans blue tracer extravasation revealed that BK-induced increase in vascular permeability was significantly increased in *VASP*$^{-/-}$ over WT mice (4.9 vs. 3.2-fold of NaCl; n=7; $p < 0.05$; Figure 14). The data indicate that VASP deficiency interferes with leakage suggesting a critical role of VASP formation for stability of tight junctions and vascular barrier integrity in BK-driven edema formation.

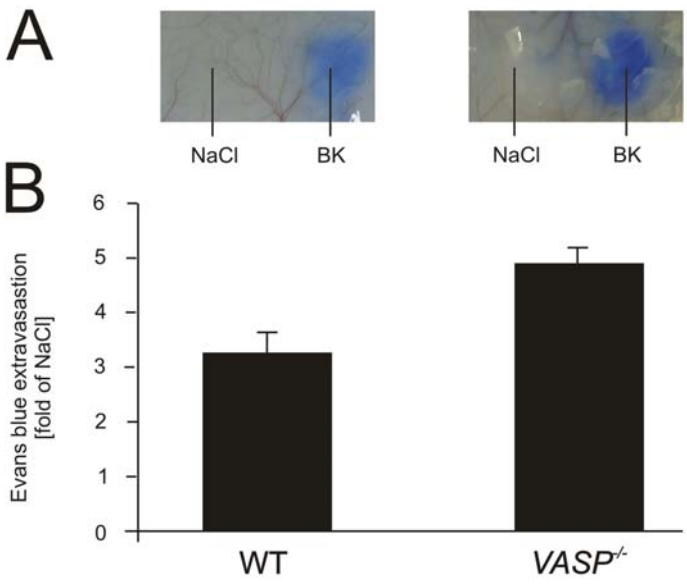

Figure 14. Function of VASP for endothelial barrier integrity. Evans blue tracer was intravenously injected into wild-type (WT) and VASP deficient (*VASP*$^{-/-}$) mice. Skin edema formation was induced by intradermally injection of bradykinin (BK). NaCl was used as a control (A). After 30 min edema sites were excisted and the fluorescence of extravasated Evans blue was quantified. Values are blotted relative to NaCl injection. Colums show means ± SEM, n=7 per group (B) (Benz et al., 2008).

[16] This text section is taken from the publication Oschatz et al., 2011.

5.6 Allergen-stimulated mast cells induce bradykinin-mediated edema

To further evaluate the role of the plasma contact system for MC-mediated skin edema under pathophysiological conditions, a model for passive cutaneous anaphylaxis, an IgE-dependent hypersensitivity reaction was set up (Siebenhaar et al., 2008). Histamine was applied as positive control and induced comparable leakage in the skin of WT, *F12*$^{-/-}$, *Bdkrb2*$^{-/-}$, *Kit*$^{W\text{-}sh/W\text{-}sh}$, and *Serping1*$^{-/-}$ mice (4.4 ± 0.5 - 4.8 ± 1.0-fold; Figure 15). In contrast, injected Hase-treated heparin failed to increase leakage over NaCl levels (1.1 ± 0.4 - 1.3 ± 0.4). Mice were topically sensitized by intradermal anti-DNP IgE injection, challenged 20 h later with intravenous injection of DNP-HSA and extravasated tracer was analyzed after 30 min (Figure 15). Allergen exposure increased leakage in WT animals to 26.6 ± 5.0 (Figures 15A and 15F), whereas *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were resistant to IgE/Ag-mediated leakage (1.2 ± 0.4; Figures 15D and 15F), supporting the specificity of the stimulus for MC-driven edema. Under the same conditions, edema was reduced to 14.6 ± 5.2 and 13.6 ± 6.6 in *F12*$^{-/-}$ and *Bdkrb2*$^{-/-}$ mice, respectively (Figures 15B, 15C and 15F). The cutaneous anaphylaxis model is consistent with data from C48/80-stimulated MC and supports a critical role of BK for sensitized MC-driven leakage.[17]

[17] The whole text of this page is taken from the publication Oschatz et al., 2011.

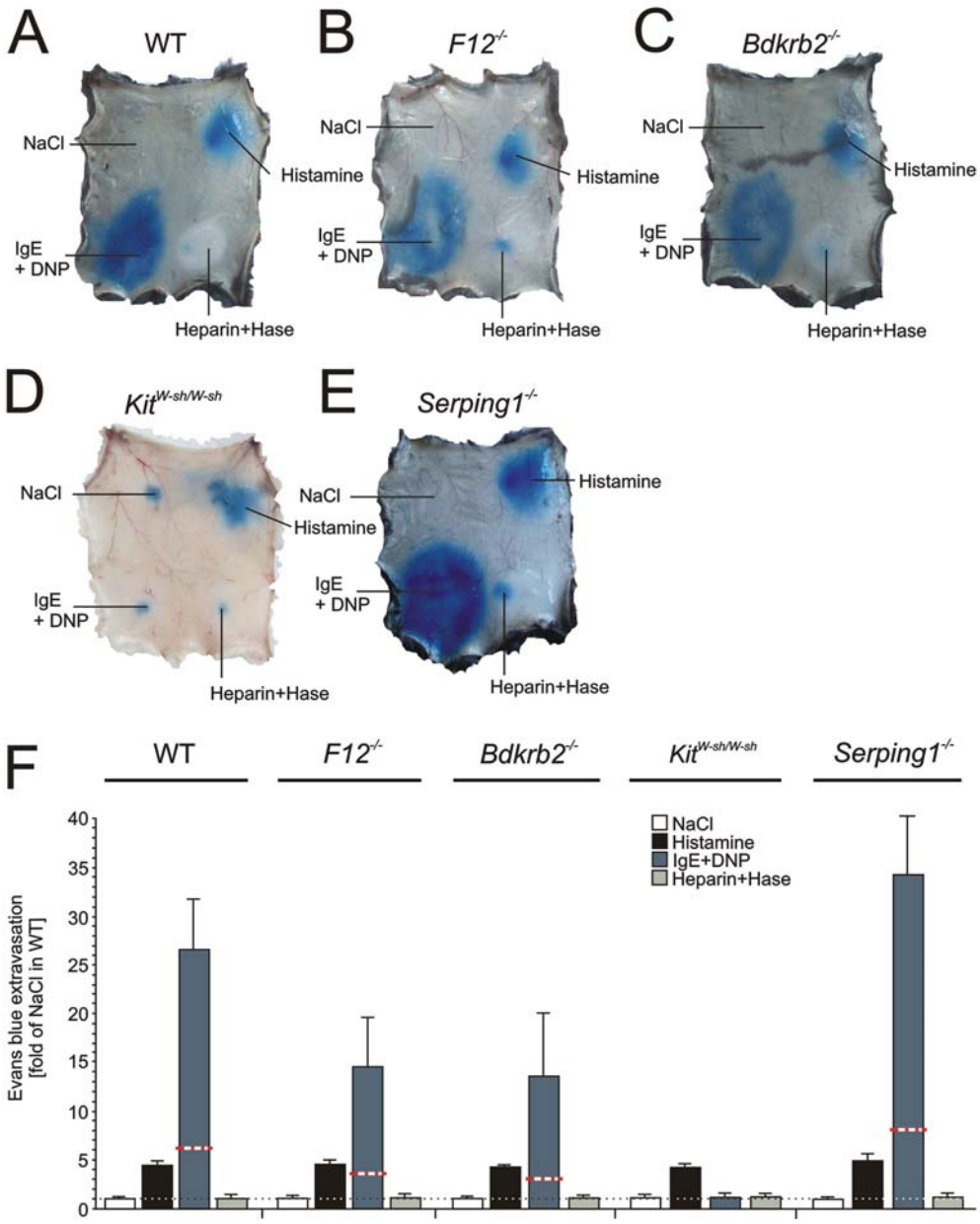

Figure 15. **BK contributes to edema in a mouse model of passive cutaneous anaphylaxis.** Mice were intradermally injected with IgE or NaCl and challenged 20 h later by intravenous injection of DNP-HSA and Evans blue tracer. The extravasation of Evans blue tracer dye after 30 min is shown in representative skins from WT, *F12*$^{-/-}$, *Bdkrb2*$^{-/-}$, *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Serping1*$^{-/-}$ mice (A-E). Tracer was extracted from the entire edema site, tracer fluorescence was quantified and signal is plotted relative to NaCl-induced extravasated Evans blue in WT mice (Red-white dashed lines represent tracer intensity in a circle of 1.2 cm diameter within the edema). Means ± SD, n=10 per group are shown (F) (Oschatz et al., 2011).

5.7 Allergens exacerbate edema in C1 esterase inhibitor deficient mice and in Hereditary angioedema patients

To analyze allergen-stimulated MC for initiating edema in HAE, *Serping1*$^{-/-}$ mice were challenged in the skin anaphylaxis model. IgE/Ag-activated MC provoked excessive edema in *Serping1*$^{-/-}$ animals (34.1 ± 6.1; $p > 0.05$ *vs.* WT; Figures 15E and 15F). As the mouse HAE model identifies allergen-sensitized MC as an initiator of pathological edema formation, the importance of this concept for the human disease state was further tested. In a survey, 38 well-characterized HAE patients from Switzerland were asked to report potential triggers of edema attacks. In 11 HAE patients, the onset of swelling was associated with allergic reactions to food and in 4 individuals edema formation was triggered by hyperimmune responses to insect toxins or drug allergens (Table 5).[18]

Table 5. HAE patients were asked to self-report potential trigger factors for their swelling attacks. Identified allergens and affected cases in 38 patients. These data resulted from collaboration and were kindly provided by W. A. Wuillemin.

Edema trigger	Affected patients
Food	11
Insect sting	3
Drug	1

[18] This text section is taken from the publication Oschatz et al., 2011.

5.8 The murine model for hereditary angioedema type III

To set up a mouse model for HAE III a double transgenic mouse (Albumin-tTAxHAE III mouse) using the tetracycline (Tet)-regulated expression system was generated. Expression of the gene of interest (*factor 12* with the mutation 1032C→A) is shut down in the presence of Tet ("Tet-Off" System). Tetracycline controlled transactivator (tTA) is liver specific expressed under control of the albumin promoter in the Albumin-tTA transgenic mouse. The tTA stimulates the transcription of the gene of interest from a minimal promoter sequence (p_{minCMV}) combined with Tet-response element (TRE) in the HAE III transgenic mouse (Figure 16).

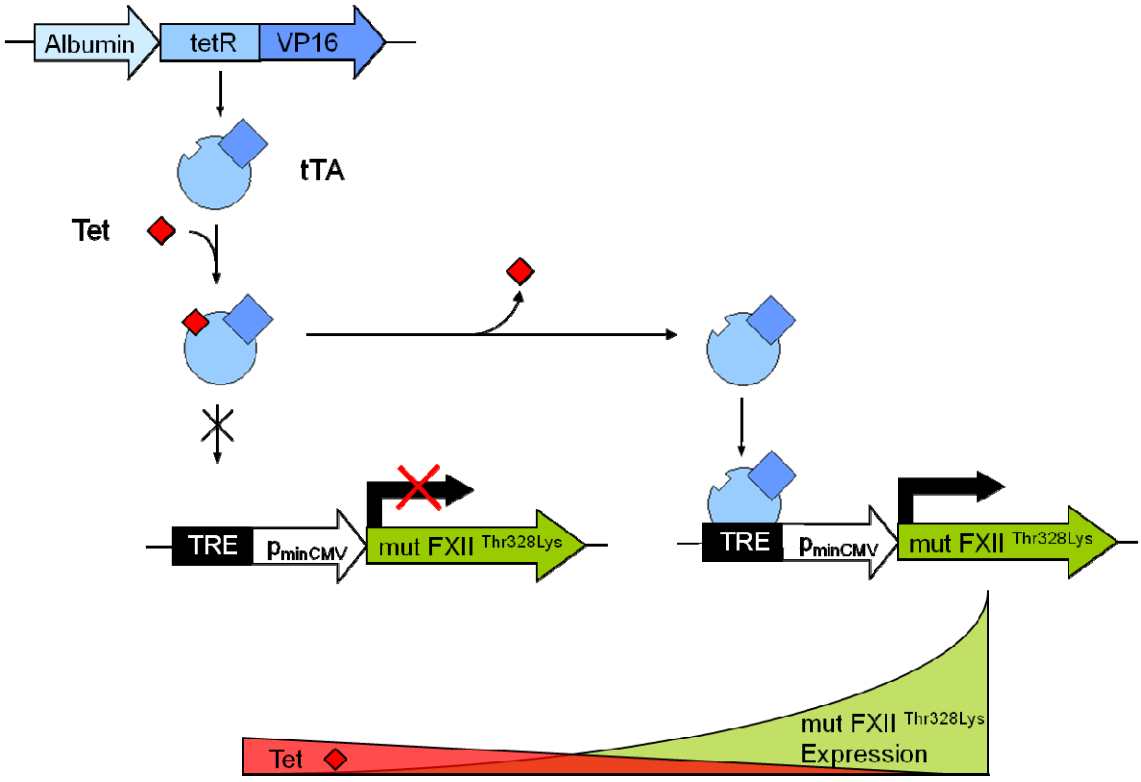

Figure 16. Liver specific inducible expression of Thr328Lys mutated human FXII by a Tet regulated system. The albumin promoter restricts expression of the regulatory protein tTA to the liver. In the absence of Tet, tTA binds to TRE-sequence and activates the expression of Thr328Lys mutated human FXII protein.

After oocytes injection of pTRE-Tight_FXIIThr328Lys 31 pups were born. Three of them carried the HAE III transgene. One HAE III transgenic male was breed with two female Albumin-tTA mice. Albumin-tTA mice were a kind gift from Dr. T.J. Liang from

the National Institutes of Health, Bethesda, Maryland, USA (Manickan et al., 2001). The two resulting litters contain 17 pups: 3/17 (18%) of the pups were HAE III transgenic, 2/17 (12%) were Albumin-tTA transgenic and 6/17 (35%) were double transgenic. 6/17 (35%) were males and 11/17 (65%) were females. Pups were phenotypically normal and fertile. For embryotransfer HAE III transgenic males were breed with C57BL/6 females. From the born pups 22/38 (58%) were HAE III transgenic. HAE III transgenic animals were mated with each other. The resulting litters were from normal size: 7-8 pups, which were phenotypically normal and fertile. Sex distribution was 45/106 (42%) male and 61/106 (58%) female. 95/106 (90%) of all pups were HAE III transgenic. Crossbreeding of embryotransfered HAE III transgenic mice with Albumin-tTA transgenic mice resulted in 62 pups. 25/62 (40%) of resulting pups were male and 37/62 (60%) were female. 1/62 (2%) mouse was HAE III transgenic, 40/62 (65%) were Albumin-tTA transgenic and 18/62 (29%) were double transgenic. The Albumin-tTAxHAE III mouse should have a Tet-regulated liver specific expression of mutated human FXII. In the presence of Tet tTA cannot bind to the TRE-sequence and mutated human FXII is not expressed. If Tet is removed, then tTA binds to the TRE-sequence and allows for the expression of the mutated human FXII (Figure 16, in our model, the Tet-derivative doxycycline instead of Tet is used and it is fed to the mice).

The mouse model for HAE III will be used in future to address the function of Thr328Lys mutated FXII for vascular leakage and edema formation *in vivo*. Therefore it is planned to crossbreed *F12*$^{-/-}$ mice with Albumin-tTA and in following steps with HAE III mice according to the breeding scheme in Figure 17. The resulting animals will possess an inducible mutated human FXII expression on the background of heterozygote endogenous FXII level. This murine model mimics the situation in HAE type III patients and allows analyzing dose-dependent effects of mutated FXII *in vivo.* It is planned to provoke edema by increasing mutant FXII expression.

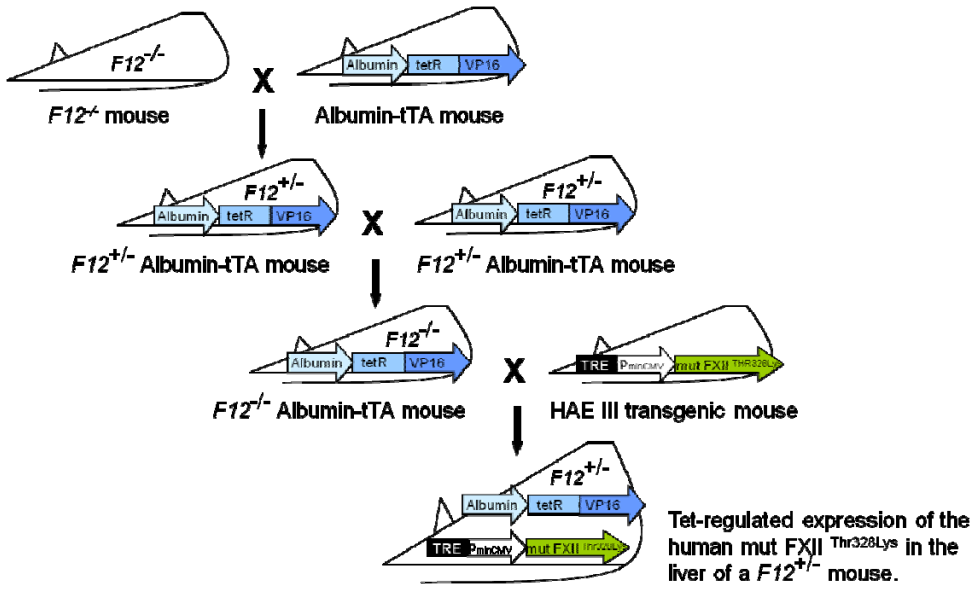

Figure 17. Generation of a mouse model for hereditary angioedema type III. *F12*$^{-/-}$ mice will be breed with Albumin-tTA mice expressing the tTA specifically in the liver. The pups will be heterozygous for *F12* (*F12*$^{+/-}$). A part of them will be Albumin-tTA transgenic. *F12*$^{+/-}$ Albumin-tTA transgenic mice will be mate resulting in animals, which are *F12*$^{-/-}$ Albumin-tTA transgenic. The animals will be breed with HAE III transgenic mice which have the TRE and are transgenic for the mutated FXII. All resulting pups will be *F12*$^{+/-}$. Some of them will be double-transgenic for tTA and Thr328Lys mutated FXII. The Albumin-tTAxHAE III transgenic *F12*$^{+/-}$ mice will have a tetracycline-regulated liver-specific expression of the human mutated FXII.

In summary, the data identify MC-heparin as an *in vivo* initiator of the proinflammatory contact activation system and demonstrate that heparin-driven BK formation, mediated by VASP, is an important component of MC-evoked vascular leakage. These findings suggest that heparin release may have broad clinical relevance in a variety of edema diseases associated with abnormal MC activation such as angioedema, urticaria or allergy.[19]

[19] This text section is taken from the publication Oschatz et al., 2011.

6 DISCUSSION

Increase in vascular permeability is characteristic for MC activity in allergic reactions and host defense is. It was suggested that MC histamine is the major mediator for leakage formation. Histamine receptor antagonists are used in therapy to treat edema formation associated with abnormal MC activity (Galli et al., 2008). BK, as well, is involved in increasing vascular permeability and is formed in allergic disease (Proud et al., 1983; Proud and Kaplan, 1988; Cicardi et al., 2010). MC-derived heparin, a highly sulfated polysaccharide, triggers BK formation via contact system activation *in vitro* (Hojima et al., 1984; Brunnee et al., 1997; Noga et al., 1999). Heparin has been used in clinic as an anticoagulant drug for over 75 years to prevent or treat thromboembolic disorders by prevention of blood clot generation (Jorpes, 1935; Li et al., 2009). This PhD thesis shows that MC-derived heparin has the ability to stimulate the contact system activation via FXII activation leading to BK generation in MC-mediated disease.

Anaphylaxis results from the rapid degranulation of MC and can lead to death. Anaphylaxis can also be a side effect of heparin. Immediate hypersensitivity reactions of heparin are rare but can be life-threatening (Smith and Harkness, 2004; Blossom et al., 2008). Intravenous administration of heparin caused cutaneous necrosis in patients (Kelly et al., 1981). Or patient developed within minutes after subcutaneous heparin treatment tongue and lips swelling, hypotension, purities or cough (Odeh and Oliven, 1992; MacLaughlin et al., 2002; Smith and Harkness, 2004). From November 2007 through January 2008 hundreds of adverse reactions associated with heparin were observed in the United States and in Germany, and 159 deaths were reported to the Food and Drug Administration (Adam et al., 2010). Heparin produced in China and manufactured by Baxter Healthcare was identified to be the reason. Reported adverse patient reactions have included: stomach pain or discomfort, nausea, vomiting, diarrhea, decreased or low blood pressure, chest pain, fast heart rate, dizziness, fainting, unresponsiveness, shortness of breath, the feeling of a strong or rapid

heartbeat, drug ineffectiveness, burning sensation, redness or paleness of skin, abnormal sensation of the skin, mouth or lips, flushing, increased sweating, decreased skin sensitivity, headache, feeling unwell, restlessness, watery eyes, throat swelling, thirst, bleeding tendencies and difficulty opening the mouth (http://www.fda.gov/Safety/Recalls/ArchiveRecalls/2008/ucm112384.htm, (Kishimoto et al., 2008)). Vials that caused reported reactions were analyzed with ^{1}H nuclear magnetic resonance spectroscopy (NMR) and capillary electrophoresis. The contaminant was identified as an unusual oversulfated form of natural chondroitin sulfate A (Blossom et al., 2008; Guerrini et al., 2008; Luhn et al., 2010). In addition, dermatan sulfate, a known impurity of heparin, was also found in selected samples (Kishimoto et al., 2008). After realizing that the heparin was contaminated the quality and safety of pharmaceutical heparin had to be ensured, but both methods require expensive equipment and are time-consuming. Therefore new tests were developed: a fluorescence assay (Luhn et al., 2010), diffusion ordered spectroscopy (Bednarek et al., 2010) and weak anion exchange high-performance liquid chromatography (Hashii et al., 2010). But the HK cleavage assay (Figures 7, 8), could also serve as a simple bioassay or might complement the currently used NMR based analysis (Guerrini et al., 2008) to screen for contaminants with high ability to activate the contact system.

It was shown that oversulfated chondroitin sulfate (OSCS) activates the contact and the complement system: OSCS-contaminated heparin activates the kallikrein amidolytic activity in human plasma. Chondroitin sulfate A was also tested and failed to induce amidolytic activity. These data confirmed the results of Hojima (Hojima et al., 1984), who showed that OSCS but not Chondroitin sulfate A, B, C activate the kinin pathway (Kishimoto et al., 2008). FXII-dependence of contact system activation by the heparin contaminate was demonstrated by the use of FXII-depleted plasma: OSCS failed to induce kallikrein-activation. OSCS induced C5a production in a bell-shape dose-dependent manner and in dependence of FXII. Pigs treated with OSCS-contaminated heparin showed hypotension (Kishimoto et al., 2008). While the group of Kishimoto

showed indirect evidence for the participation of BK in anaphylactic reaction caused by OSCS-contaminated heparin via amidolytic activity of kallikrein Adam and co-workers quantified directly the BK-release, induced by OSCS-contaminated heparin incubated with human plasma (Adam et al., 2010). In another study dextran sulfate was infused intravenously in miniature pigs, leading to profound and transient systemic arterial hypotension with concomitant reduction in kininogen. Dextran sulfate-induced hypotension was completely blocked by HOE-140 (Icatibant)-pretreatment. However kininogen was depleted, showing that hypotension is produced by kininogen cleavage and BK formation and mediated via kinin B2 receptor signaling (Siebeck et al., 1994). In this PhD thesis heparin instead of dextran sulfate was applied intra-arterially in rats leading to the same effects: rapid and pronounced blood pressure decrease (Figure 10E). The procedure was repeated in mice, followed by a decrease of arterial blood pressure (Figure 10F). It was shown that this heparin-induced hypotonic effect was mediated via activation of the contact system since *F12*$^{-/-}$ and *Bdkrb2*$^{-/-}$ mice were almost protected. Using pharmacological substances to block FXII (PCK) and B2R (Icatibant) led to the same results. These data (Figures 10E-G) indicate the role of BK in immediate adverse reactions.

Dextran sulfate is a synthetic negatively charged polysaccharide containing 4 sulfate residues per disaccharide in average. The same goes for OSCS (Guerrini et al., 2008). Heparin has 2.7 sulfate residues per disaccharide, while heparan sulfate contains only 1 sulfate residues (Capila and Linhardt, 2002). The potency to activate FXII via contact decreased from dextran sulfate and OSCS to heparin. Heparan sulfate failed to activate the contact system (Hojima et al., 1984). The quantity of BK release in human plasma in presence of dextran sulfate was higher than that measured with heparin at the same concentration (Adam et al., 2010). This implicates that the negative charge density of polysaccharide is more important rather than the defined structure for contact activation triggering BK formation. This was demonstrated in Figures 8A-F.

Anaphylactoid reactions have been also reported in patients dialyzed with a negatively charged membrane and treated with an ACE inhibitor (Tielemans et al., 1990; Verresen et al., 1990). Aminopeptidase and carboxypeptidase N are degrading enzyme of BK, but ACE is the major one. ACE inhibitors lower ACE concentration with the result that patients develop more likely life-threatening edema (Summers et al., 2008).

This PhD thesis demonstrates that IgE/Ag challenge led to MC degranulation with increased heparin activity in mice (triggering of HK cleavage Figure 10G and triggering of edema formation Figure 15). In *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice IgE/Ag challenge failed to increase vascular permeability, as shown before by Zhou et al. (Zhou et al., 2007) (Figure 15D). IgE/Ag-challenged mice had plasma that does not clot because of released heparin of >4 µg/mL. This concentration already induced BK formation (Figure 7). The animal model data are consistent with anaphylactic reactions in humans. Patients with anaphylaxis or anaphylactic shock due to insect bites show a significantly prolonged aPTT and an increased prothrombine time (Mazzi et al., 1994; Wang et al., 2005; Lombardini et al., 2009). The prolonged aPTT during anaphylaxis is caused due to activated MC, which release mediators such as heparin. The polysaccharide acts as an anticoagulant via binding to ATIII (Lombardini et al., 2009).

The capacity of MC-heparin to activate FXII in plasma is controversial (Hojima et al., 1984; Pixley et al., 1985a; Silverberg and Diehl, 1987; Pixley et al., 1991; Brunnee et al., 1997; Noga et al., 1999). Commercial heparin is purified from animal sources, usually bovine (in the past), porcine intestinal mucosa or lung by specific patented manufacturing processes. Since most heparins are obtained by different methods, the final products are specific in their molecular and structural compositions and so they differ in their *in vitro* and *in vivo* properties, e.g. potency of contact system activation (Fareed et al., 2004). However, it seems, that heparin has 2 different functions for the FXII activation. First, heparin is a negatively charged polysaccharide, to which FXII binds to be autoactivated. Second, heparin increases the inhibition of FXII by ATIII. The

irreversible inhibitor binds to a pentasaccharide sequence of heparin with high affinity via an internal unique 3-O-sulfated glucosamine unit (Stead et al., 1976; Meyer et al., 1981; Olson et al., 1993; Capila and Linhardt, 2002; Petitou et al., 2003). The dual function of heparin for contact system activation is indicated in a bell-shape dose response (Pixley et al., 1991; Jesty et al., 2005), which is also observed for OSCS (Kishimoto et al., 2008).

Heparin in MC granules forms tight complexes with proteases (tryptases, chymases) and histamine. It is essential to obtain proper storage of tryptase (Hallgren et al., 2001). Upon MC activation the protease/heparin complexes are exocytosed (Stevens and Adachi, 2007). Still the association of heparin and protease is required for stabilization of tryptase (Addington and Johnson, 1996). The polysaccharide is also required for autocatalytic processing of protryptase to mature tryptase monomer (Hallgren et al., 2000). To exclude that MC-derived heparin was still bound to tryptase or was contaminated heparin was purified as described.

Evidence of the importance of heparin in packing of proteases in MC granules was adduced by the work of Humphries and Forsberg. Mice with targeted inactivation of the *N-deacetylase/N-sulfotransferase-2* (*NDST-2*) gene are unable to synthesize sulfated heparin (Forsberg et al., 1999; Humphries et al., 1999) resulting in a largely decreased content of several MC proteases (tryptase, chymase) and histamine. *NDST-2* deficient (*NDST-2*$^{-/-}$) mice have fewer MC, with a reduced number of smaller granules and large empty vacuoles leading to reduced granula secretion after stimulation by IgE/Ag (Forsberg et al., 1999). These data suggest the physiological importance of heparin in MC granules for storage of other mediators and thereby for function of MC.

The combined deficiency of histamine and several other MC mediators together with heparin (Forsberg et al., 1999) argue against the use of *NDST-2*$^{-/-}$ mice to specifically analyze the impact of activated MC triggered BK formation for endothelial barrier function. Reconstitution of MC deficient mice with *NDST-2*$^{-/-}$ MC led to a difficult reproducibility of IgE/Ag-induced edema formation. Therefore *Kit*$^{W\text{-}sh/w\text{-}sh}$ mice were

employed, which are described as being MC deficient (Figures 11, 12, 15) (Grimbaldeston et al., 2005; Piliponsky et al., 2010). The IgE/Ag stimulated leakage formation in these animals was compared with WT and animals having deficiency in heparin-driven BK formation (*F12*$^{-/-}$ mice) and *Bdkrb2*$^{-/-}$ mice (that are resistant to BK signaling but susceptible for histamine mediated leakage) to specifically address the importance of activated MC triggered BK formation for vascular permeability.

Humans with decreased level or functional defect of C1INH have HAE type I or II, whereas HAE type III is associated with a single point mutation in *factor 12* gene (Cichon et al., 2006). HAE is characterized by recurrent attacks of angioedema and BK is the key mediator of symptoms (Nussberger et al., 2002; Cicardi et al., 2010). But the swellings are not associated with an increased pro-thrombotic risk (Nzeako et al., 2001). This supports the concept of selective activation mechanisms for individual contact-system initiated pathways. Patients (Nussberger et al., 1998; Zuraw, 2008) and the *Serping1*$^{-/-}$ mice (Figure 11, 12E) have normal vascular permeability under non-stimulated conditions. This is in contrast with C1INH deficient mice, generated by Han, which have aconstantly increased vascular permeability under non-stimulated conditions (Han et al., 2002). The different phenotypes of the two mouse models can be caused by different technologies used to inactivate the *Serping1* gene. The *Serping1*$^{-/-}$ mice were generated via random mutagenesis, which deleted exon 2, resulting in complete termination of C1INH protein synthesis (Figure 4). In contrast Han and coworkers used a random insertional mutagenesis technique (gene trap), in which the trapping vector is inserted into a splice-acceptor site in intron 6 (210 bp 5´of exon 7). Therefore *Serping1* gene expression is not complete, but the C1INH protein is truncated (composed of exons 1-6, amino acids 1-347 of 504 in the mature protein, accession no. AAC40149) and fused to trap-vector, which codes β-galactosidase (Ullrich and Schuh, 2009). The truncated C1INH lacks the active site (encoded by exon 8), which includes the signal peptide in WT C1INH. Therefore the mutant could be

secreted into the plasma. In any case, it affects the permeability leading to increased basal permeability. So the biological function and location has to be determined. In Western Blot analyses Han showed only the full-length C1INH protein proving the deficiency of C1INH. But he forgot to show the size in which the truncated variant is supposed to be.

While the *Serping1*$^{-/-}$ mice is a model for HAE II, the double transgenic mouse (Albumin-tTAxHAE III mouse) line, described in this PhD thesis, is a model for HAE III. Analyses of the double transgenic mice need to be done to show that the mouse line exhibits the conditional expression of 1032C→A point mutated human FXII in the liver. Expression of mutated FXII could be tested via Western blotting of plasma samples or digested liver-tissue of Albumin-tTAxHAE III mice with the α-FXIIHAE antibody, which exists in the laboratory group. Additional it has to be demonstrated, that the expression of the HAE III transgene could be completely suppressed by Tet administration and that such a suppression could be reversed upon discontinuation of Tet. The biological significance of this system has to be confirmed by phenotypic studies, in which the double transgenic animals develop angioedema triggered by to investigative stimuli in the absence of Tet.

Several transgenic mouse models have been developed using the Tet-regulated system in various target tissues or organs: in lymphoid cells, cardiac muscles and rat liver (Gossen and Bujard, 1992; Passman and Fishman, 1994; Manickan et al., 2001). For the HAE III model it was reasonable to adopt a liver specific expressed tTA under control of the albumin promotor, because both, albumin and FXII are produced in the liver. Therefore the human mutated FXII, which could be inserted in the DNA of all body cells, is only expressed in the liver, where the tTA is synthesized in the absence of Tet. A Tet-Off system was applied instead of mutation techniques in *factor 12* gene, because the Tet -regulated system allows analyzing not only for a switch on-switch off expression, but also for a dose-dependent effect of mutated FXII *in vivo.*

Following contact-induced activation, FXIIa initiates several protease cascades in plasma: the complement and fibrinolytic system, kallikrein-kinin system and the intrinsic pathway of coagulation (Muller and Renne, 2008; Oschatz et al., 2011). Using genetically modified mice, it was shown that FXIIa-initiated fibrin formation is essential for arterial thrombus formation (Renne et al., 2005a) and ischemia reperfusion injury in an experimental model of stroke (Kleinschnitz et al., 2006). Polyphosphates secreted by platelets activate FXII. FXIIa activates, via its substrate FXI, the intrinsic pathway of coagulation and, via PK, the kallikrein-kinin system. Heparin, in contrast, leads only to FXII-mediated BK formation without FXI-triggered coagulation (Figure 7D). These data confirm the results of Kelly et al., where i.v. administration of porcine heparin leads to necrosis but histologically not to fibrin thrombi in capillaries and vessels (Kelly et al., 1981). The *in vivo* relevance of other FXIIa triggered pathways is less clear. *In vitro*, FXIIa initiates the classical complement system pathway that culminates in the generation of C3a and C5a (Ghebrehiwet et al., 1981). Both mediators contribute to anaphylactic reactions (del Balzo et al., 1989) and are thought to play a role in adverse reactions induced by contaminated heparin (Schwartz, 2008). Since B2R antagonists in rodents and genetic ablation of BK receptors in mice almost completely abolished heparin-induced adverse effects in our animal models (Figure 10E, 10F, 11, 12C and 13), FXIIa-triggered hypotension and anaphylaxis appear to be predominantly BK mediated. In a swine model, a dextran sulfate infusion-induced transient systemic hypotension that was completely blocked by the B2R antagonist Icatibant (Siebeck et al., 1994).

MC heparin, highly sulfated polysaccharides and polyphosphates are not the only *in vivo* activators of the FXII/PK-system. Recently, misfolded proteins were identified as a FXII activating surface in humans (Maas et al., 2008). Similar to heparin or dextran sulfate (Johne et al., 2006), the binding of FXII to protein aggregates specifically trigger the kallikrein-kinin pathway without activating the FXIIa substrate, FXI (Maas et al., 2008), which drives the intrinsic clotting pathway. In contrast, extracellular RNA

activates FXII, and a synthetic RNA analog is a procoagulant (Kannemeier et al., 2007). Since FXI and PK have homologous structures, and are both complexed with HK to the cell surface in plasma (Renne et al., 2002), the detailed mechanism for selective activation of FXIIa substrate PK over FXI is not completely understood but could be caused because of a higher plasma concentration of PK *vs.* FXI, different FXII-activation mechanisms and different FXIIa-forms (Schmaier, 2008; Renne, 2012).

In conclusion, this PhD thesis offers arguments for the immediate adverse heparin effects and for the 90-year-known phenomenon, that MC-driven anaphylaxis is associated with impaired plasma clotting (Bulger, 1918): that now can be explained by negatively charged MC heparin. The polysaccharide activates FXII-dependent the plasma contact system leading to BK formation with pathological MC-mediated inflammation, hypotension and edema formation.

7 CONCLUDING REMARKS

MC have a central function in allergic and inflammatory diseases and contribute to increased vascular permeability, allergic and anaphylactic reactions. Upon activation MC release a wide range of mediators. One of them is heparin, which is commonly used as an anticoagulant drug.

This study identifies a mechanism, by which MC-heparin increases vascular permeability. The negatively charged polysaccharide initiates FXII-driven contact activation. FXII triggers the kallikrein-kinin system leading to BK formation. The inflammatory mediator induces swellings, anaphylactic and inflammatory symptoms, which are known to be associated with aberrant MC activity. Thus, the study provides a novel link between MC-driven pro-inflammatory reactions and the kallikrein-kinin system (Figure 18). Drugs that block BK or FXII activity protect from adverse MC-driven effects in patients and genetically engineered mouse models and could be a new strategy to treat MC-driven allergic diseases.

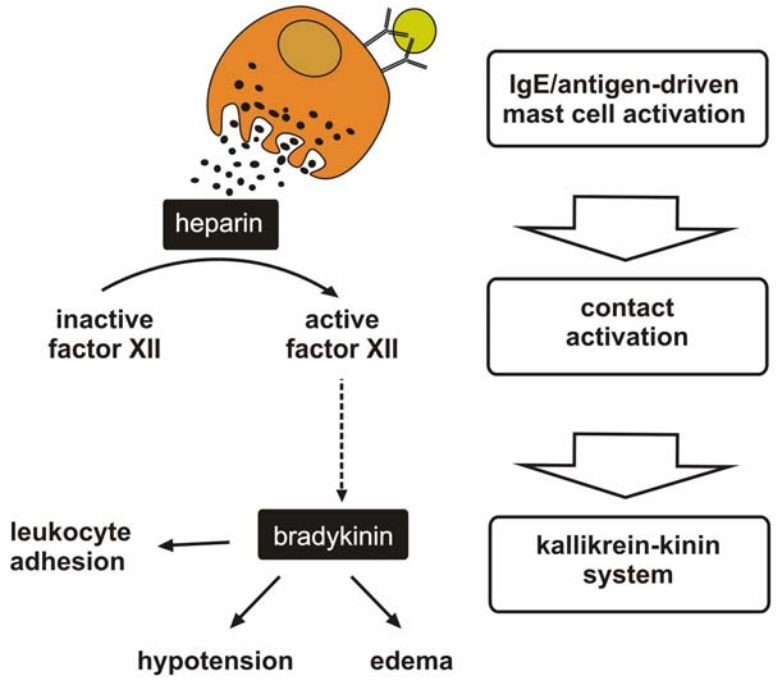

Figure 18. Mast cell-released heparin increases vascular permeability *in vivo*: a novel link between mast cell-mediated diseases and the kallikrein-kinin system. This figure has been published before - online as a graphical abstract to (Oschatz et al., 2011).

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9 APPENDIX

Abbreviations

α	anti
µ	micro
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
ACE	angiotensin-converting enzyme
Ag	antigen
aPTT	activated partial thromboplastin time
ATIII	antithrombin III
BK	bradykinin
bp	base-pair
B2R	kinin B2 receptor
BSA	bovine serum albumin
bw	bodyweight
C1INH	C1 esterase inhibitor
C48/80	compound 48/80
Da	Dalton
dNTP	deoxyribonucleotide
ELISA	enzyme linked immunosorbentassay
FITC	Fluorescein isothiocyanate
FX	plasma coagulation factor X
FXa	activated plasma coagulation factor X
FXI	plasma coagulation factor XI
FXII	plasma coagulation factor XII
FXIIa	activated plasma coagulation factor XII
g	gram
g	gravity
h	hours
HAE	Hereditary angioedema
HK	high molecular weight-kininogen
IgE	immunoglobulin E
IgG	immunoglobulin G
ITAM	immunoreceptor tyrosine–based activation motifs

i.p.	intraperitoneal
i.v.	intravenous
k	kilo
L	liter
LK	/ molecular weight-kininogen
M	Molarity (mol/L)
mA	miliampere
mAb	monoclonal antibodies
MC	mast cells
mg	miligramm
min	minutes
mL	mililiter
mm	millimeter
mM	millimolar
mmHg	millimeter of mercury
MW	molecular weight
ng	nanogramm
nm	nanometer
nM	nanomolar
NO	nitric oxide
NP	normal plasma
pAb	polyclonal antibodies
PAGE	polyacrylamide gel
PCK	H-D-Pro-Phe-Arg-chloromethylketone
PCR	polymerase chain reaction
pH	negative base-10 proton concentration
PK	plasmakallikrein
PKA	cyclic adenosine monophosphate-dependent protein kinase
PKC	protein kinas C
PKG	cyclic guanosine monophosphate-dependent protein kinase
PPK	plasma prekallikrein
PPP	platelet pure plasma
OD_{600}	optical density at a wavelength of 600 nm
OSCS	over-sulfated chondroitin sulfate
rpm	revolutions per minute
RT	room temperature
SEM	standard error of the mean

SD	standard deviation
SDS	sodium dodecyl sulfate
sec	seconds
Tet	tetracycline
tetR	tet repressor protein
TRE	tetracycline-response element
tTA	tetracycline transactivaor
U	unit
UV	Ultraviolet
V	Volt
VASP	vasodilator-stimulated phosphoprotein
vs.	versus
w/o	without
WT	wild-type

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Publications

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2. Kilinç, E., Van Oerle, R., Borissoff, J.I., **Oschatz, C.**, Gerlofs-Nijland, M.E., Janssen, N.A., Cassee, F.R., Sandström, T., Renné, T., Ten Cate, H., and Spronk, M.H. (2011). Factor XII activation is essential to sustain the procoagulant effects of particulate matter. J. Thromb. Haemost. *9*, 1359-67.

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Presentations

Presentations with published abstracts

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4. **Oschatz, C.**, Lecher, B., Müller-Esterl, W., Walter, U., and Renné, T. (2007) Mast cell heparin induces acute inflammations by bradykinin formation *in vivo*. Joint Conference of the Austrian Society for Laboratory Medicine and Clinical Chemistry and the German Society for Clinical Chemistry and Laboratory Medicine, Vienna, Austria, September 19-22, 2007. P129 [Poster]

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Affidavit

I hereby confirm that my thesis entitled “Mechanisms and functions of the mast cell-activated contact system in inflammatory reactions” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Mechanismen und Funktionen des Mastzell-aktivierten Kontaktsystems für Entzündungsreaktionen“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift