Wechselwirkungen zwischen humanen Darmmastzellen und humanen Darmfibroblasten

(Interactions between human intestinal mast cells and human intestinal fibroblasts)

Universität Hohenheim

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Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften (Dr.rer.nat)

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Vorgelegt von
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For my parents

Zusammenfassung

Fibroblasten spielen eine zentrale Rolle in der Pathogenese von Fibrose, da sie die Hauptquelle der extrazellulären Matrixproteine sind. Allerdings ist die Regulation der Fibroblasten bei der Bildung der extrazellulären Matrix, der Mechanismus der zum Kontrollverlusst der extrazellulären Matrix Homeostase bei der chronischen Entzündung führt, und die Funktion, die humane Darmmastzellen dabei spielen, noch nicht verstanden.

Mastzellen besitzen eine Schlüsselrolle bei allergischen Reaktionen, sind aber auch an der Immunabwehr, bei Gewebeneubildungsprozessen wie z.B. der Wundheilung, der Angiogenese und der Fibrogenese beteiligt. Die Arbeitgruppe von Prof. Bischoff konnte bereits zeigen, dass humane Darmfibroblasten Apoptose in humanen Darmmastzellen unabhängig von den bekannten Mastzell-Wachstumsfaktoren Stem Cell Factor, IL-3, IL-4 und Nerve Growth Factor unterdrücken. In meiner Arbeit konnte ich nun zeigen, dass die Effekte von Fibroblasten auf Mastzellen von IL-6 vermittelt werden. Es wurden die molekularen Interaktionen zwischen humanen Mastzellen und humanen Fibroblasten, beide isoliert und aufgereinigt aus Darmgewebe, untersucht. Das Überleben der Mastzellen bei Anwesenheit von Fibroblasten konnte mit einem anti-IL-6 Antikörper verhindert werden. Mastzellen, die mit IL-6 inkubiert wurden, überlebten bis zu 3 Wochen, genauso wie Mastzellen, die mit Fibroblasten co-kultiviert wurden. Stimuliert durch die Co-Kultivierung mit Mastzellen oder durch Mastzellmediatoren, produzieren Darmfibroblasten IL-6. Außerdem bilden Fibroblasten nach Stimulation mit Mastzellmediatoren das antifibrotische Enzym Matrixmetalloproteinase-1. Matrixmetalloproteinase-1 wird als multifunktionelles Molekül betrachtet, da es nicht nur am Umsatz der Collagenfasern in der extrazellulären Matrix beteiligt ist, sondern auch an der Teilung zahlreicher “Nicht-Matrix“-Substrate und Zelloberflächenmoleküle, weshalb ihm eine Rolle bei der Regulation der Zellfunktionen zugeschrieben wird. Erstaunlicherweise verlieren Fibroblasten, die mit Mastzellen co-

kultiviert, oder mit Matrixmetalloproteinase-1 behandelt werden, ihre Konfluenz. Die von Mastzellen in Fibroblasten ausgelöste Matrixmetalloproteinase-1 Expression, hängt von dem MEK/ERK Signalweg ab, wie unsere Inhibitionsexperimente zeigten.

Zusammenfassend kann gesagt werden, dass die vorliegende in vitro Studie zeigt, dass Mastzellmediatoren Fibroblasten stimulieren IL-6 zu bilden und umgekehrt die Bildung von IL-6 durch Fibroblasten das Überleben der Mastzellen fördert. Außerdem induzieren Mastzellmediatoren die Expression von Matrixmetalloproteinase-1 in Fibroblasten. Die Ergebnisse meiner Arbeit deuten darauf hin, dass Mastzellen, die an fibrotischen Stellen akkumulieren, Fibrogenese eher unterdrücken als Fibrogenese unterstützen.

Summary

Fibroblasts (FB) play a central role in the pathogenesis of fibrosis since they are the major source of extracellular matrix proteins. However, the regulation of extracellular matrix production in fibroblasts, the mechanisms that lead to loss of control of extracellular matrix homeostasis during chronic inflammation and the role of human intestinal mast cells are still not fully understood.

Mast cells are key effector cells in allergic reactions but also involved in host defense and tissue remodeling processes such as wound healing, angiogenesis, and fibrogenesis. The group pf Prof. Bischoff has shown previously that human intestinal fibroblasts suppress apoptosis in human intestinal MC independent of the known human mast cell growth factors stem cell factor interleukin-3, interleukin-4, and nerve growth factor.In this work I could show that the effects of fibroblasts on mast cells are mediated by interleukin-6. The molecular crosstalk between human mast cells and human fibroblasts, both isolated and purified from intestinal tissue was analyzed. Mast cells survival in the presence of fibroblasts could be blocked using an anti-interleukin-6 antibody. Mast cells incubated with interleukin-6 survived for up to 3 weeks. Intestinal fibroblasts produced interleukin-6 upon direct stimulation by mast cells in co-culture or by mast cell mediators such as tumor necrosis factor alpha, interleukin-1 beta, tryptase or histamine. Moreover, fibroblasts stimulated by mast cell mediators produce the antifibrotic enzyme matrix metalloproteinase-1. Matrix metalloproteinase-1 should be considered as multifunctional molecule since it participates not only in the turnover of collagen fibrils in the extracellular space but also in the cleavage of a number of non-matrix substrates and cell surface molecules suggesting a role in the regulation of cellular behaviour. Noteworthy, fibroblasts co-cultured with mast cells or treated with matrix metalloproteinase-1 lost confluence. Matrix metalloproteinase-1 expression in

fibroblasts triggered by mast cells was dependent on the MEK/ERK cascade as shown by inhibitor experiments.

In conclusion, this study show that mast cells mediators stimulate fibroblasts to produce interleukin-6, and, vice versa, fibroblasts derived interleukin-6 supports mast cells survival. Furthermore, mast cell mediators induce expression of matrix metalloproteinase-1 in fibroblasts, a key enzyme in fibrolysis, which in turn leads to lost of confluence of cultured fibroblasts. Taken together the results of my work suggest that mast cells accumulating at sites of fibrosis rather limite than promote fibrogenesis.

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Abbrevations/Abkürzungsverzeichnis

Ab	antibody
Ag	antigen
cd	cluster of differentiation
CD	Crohn´s disease
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTP	2´-deoxynucleoside5´-triphosphate
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FB	fibroblasts
FcR	Fc receptors
FCS	fetal calf serum
g	gram (only with numbers)
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
G-CSF	granulocyte CSF
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage CSF
GTP	guanosine triphosphate
h	hour (only with numbers)
IEC	intestinal epithelial cells
IFN	interferon
Ig	immunoglobulin

IL	interleukin
HSR	kinase suppressor of Ras
mAb	monoclonal Ab
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MC	mast cells
MC_T	tryptase positive and chymase negative mast cells
MC_{TC}	tryptase and chymase psotive mast cells
MEK	MAPK kinase
mg	milligram (only with numbers)
min	minute (only with numbers)
ml	milliter (only with numbers)
mRNA	messenger RNA
µg	microgram (only with numbers)
µl	microliter (only with numbers)
n	*number in study*
NS	not significant
OD	optical density
OVA	ovalbumin
p	probability
P	phosphorylation
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PP2A	protein phosphatase 2A
RKIP	raf kinase inhibitor protein

RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
s	second (only with numbers)
SMA	smooth-muscle cell actin
SMC	smooth-muscle cell
SRF	serum response factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
U	unit (only with numbers)
UC	ulcerative colitis
UV	ultraviolet
v/v	volume to volume ratio (%)
W	watt (only with numbers)
wk	week (only with numbers)

Introduction

1.1 Mast cells

1.1.1 Characteristics of mast cells

MC were known as a key cell type involved in type I hypersensitivity (2). Until last two decades, this cell type was recognized to be widely involved in a number of non allergic diseases in internal medicine including chronic obstructive pulmonary disease, Crohn´s disease, fibrosis, liver cirrhosis, and cardiomyopathy (3) (Table 1).

Table 1: Mast cells and non allergic diseases

Disease	MC hyperplasia	Release MC mediators
Chronic obstructive pulmory disease	Yes	Yes
Cor pulmonale	Yes	No
Bronchectasis	Yes	Yes
Acute respiratory distress syndrome	Yes	Yes
Bronchiolitis obliterans organizing pneumonia	Yes	Yes
Cystic fibrosis	Yes	Yes
Intestitial lung disease	Yes	Yes
Silicosis	Yes	No
Sarcoidosis	Yes	Yes
Gastritis	Yes	Yes
Ulcerative colitis	Yes	Yes
Crohn´s disease	Yes	Yes
Liver cirrhosis	Yes	No
Hepatitis	Yes	No
Pancreatitis	Yes	Yes
Atherosclerosis	Yes	No
Myocardial infarction	Yes	Yes
Congenital heart disease	Yes	No
Myocarditis	Yes	No
Cardiomyopathy	Yes	Yes
Diabetes	Yes	No
Thyroiditis	Yes	No
Osteoporosis	Yes	No
Glomerulonephritis	Yes	No
Nephropathy	Yes	No
Multiple sclerosis	Yes	No

Modified from He (3)

MC are round or oval cells with an unlobed nucleus that are found in many tissues such as the skin and at mucosal sites where they preferentially locate around blood vessels and nerves. MC derive from CD34+ hematopoietic progenitor cells. Previous studies (4, 5) suggested that bone marrow derived MC progenitors circulate in the peripheral blood and subsequently migrate into the tissue where they undergo final maturation under the influence of local microenvironmental factors. The regulation of this process and the stage of maturation at which MC migrate from the blood into the tissue remain largely unknown. Under normal conditions cells expressing typical markers of mature MC are not found in peripheral blood. Human MC are commonly classified according to their protease contents. MC containing tryptase only (MC_T) predominate in the lung and intestinal mucosa. Tryptase and chymase positive MC (MC_{TC}) are mainly located in the skin and the intestinal submucosa (6, 7).

Stem cell factor (SCF) has been described as an essential factor for both MC maturation and maintenance of mature MC in the body since it induces MC proliferation and suppresses MC apoptosis. The importance of SCF and its receptor *c-kit* is stressed by the fact that SCF- or c-kit-deficient mice basically lack MC, even though in vitro IL-3 is also capable of inducing partial MC development in rodents, but not in humans. Most studies have shown that human MC development from progenitor cells and growth of mature tissue derived MC is essentially SCF-dependent (8).

1.1.2 Role of mast cells in physiology

1.1.2.1 Blood flow and coagulation

MC have been associated with bleeding in a variety of disorders. In cutaneous mastocytosis, for example, gastrointestinal and cutaneous bleeding has been attributed to the heparin released by MC (9). Skin mast cells have also been shown to prolong the bleeding

time and to inhibit thrombin generation (10). The clinical association between mast cells, bleeding, and fibronolysis has led some investigators to propose recently that mast cells may play a physiologic role in promoting a profibrinolytic and antithrombotic state within injured tissues (11).

One possible mediator of this anticoagulant activity is mast cell tryptase (12). The serine protease tryptase is the major protein component of human mast cell secretory granules, where it exists in a complex with heparin proteoglycan (13). Both heparin and tryptase are found only in mast cell granules and are tightly to one another under physiologic conditions (13). Recent evidence indicates that there is a structural requirement for heparin to bridge tryptase monomers to form enzymatically active tryptase tetramers (14).

The physiologic substrate for tryptase remains unknown. There is evidence, however, that mouse mast cell tryptase exhibits anticoagulant activity in vivo and in vitro due to its ability to degrade fibrinogen (15). Studies with human mast cell tryptase renders fibrinogen unclottable by thrombin (16).

1.1.2.2 Smooth-muscle contraction and peristalsis of the intestine

Histamine and various Leukotrienes (LTC_4 and LTD_4) are the classic mediators of MC, causing contraction of smooth muscle (SM) via their respective receptors (17). Platelet-activating factor and PGD_2 are also released and can cause contraction (18-20). The effect of PGE_2 is biphasic; at low concentrations, PGEs relaxes SM, and at higher concentration causes contraction via the thronboxane A_2 receptor. In addition to the direct contractile effects on the muscle, two MC mediators, TNF-α and tryptase, have both been shown to induce hyperresponsiveness of SM.

The mediators of MC degranulation all cause a contractile response or induce hyperresponsiveness in SM, with only one mediator, PGE_2, capable of causing both contraction and relaxation of SM depending on its concentration. Thus, in terms of effects on

SM contraction, MC mediators may cause contraction or relaxation, but the evidence suggests that the majority of mediators are stimulatory.

Nematode infections are accompanied by increases in intestinal muscle contractility and propulsive activity (21-24) as well as accelerated intestinal transit (25). During such infections, the intestinal motor apparatus acts as an extension of the immune system aiding in the expulsion of pathogens through increased propulsive activity. Because mice that are MC deficient were not able to clear nematode efficiently (26).

1.1.2.3 Mucosal secretion

MC signal the presence of the antigen to the enteric nervous system, which uses one of the specialized programs from its library of programs to remove the antigens. This is accomplished by stimulating mucosal secretion, which flushes the antigen into the lumen and maintains it in suspension. The secretory response then becomes linked to powerful propulsive motility, which propels the secretions together with the offending agent rapidly in the anal direction (27).

1.1.2.4 Wound healing

MC are implicate in three phases of wound healing: the inflammatory reaction, angiogenesis and extracellular-matrix reabsorption. The inflammatory reaction is mediated by released histamine and arachidonic acid metabolites. Compound 48/80 and disodium-cromoglycate are both able to increase skin breaking strength shortly after wounding. Under light and electron microscopy, Trabucchi *et al* (28) found that small, granule-poor, irregular MC mucosal-like mast cells (MLMC) accumulate in the wound. This suggests that the small MLMC migrate into the skin during wound healing, and that both connective-tissue mast cells (CTMC) and MLMC are involved in tissue repair. Moreover, there is some evidence that MC participate in angiogenesis, since heparin is able to stimulate endothelial-cell migration and proliferation in vitro, and protamine to inhibit these processes and also angiogenesis in vivo. Further studies are needed to demonstrate that protamine is specifically involved in inhibiting

heparin-mediated angiogenesis in wounded tissue. Finally, mast cells may play a role in the extracellular matrix remodelling, on the basis of *in-vitro* experiments but there are still no *in-vivo* data.

1.1.2.5 Regulation of innate and adaptive immune responses

MC have an action on dendritic cells (DC) for the migration, maturation and function. TNF and IL-1 can facilitate the migration and functional maturation of DC(29, 30), and other potential MC products, including IL-16 (31), IL-18 (32), CCL5 (33-36) and prostaglandin E2 (37) can also promote DC migration. MC derived TNF and exosomes can upregulate expression of $\alpha_6\beta_4$ and $\alpha_6\beta_1$ integrins, MHC class II, CD80, CD86 and C, thereby promoting functional maturation of DC (38). Histamine can enhance expression of MHC class II and costimulatory molecules on DC though both H1 and H2 receptors (39), whereas histamine has no effect on LPS-induced DC maturation (40). However, both histamine (41) and prostaglandin D_2 or prostaglandin E_2 (42, 43) can inhibit IL-12 production by DC and induce maturation of DC toward an effector DC2 phenotype, leading to the polarization of naïve T cells to Th2 cells.

MC cells can occur in close proximity to T cells at sites of allergic reactions and in other immunological responses (44), and MC can promote T cell migration either directly, by producing chemotactic factors such as IL-16, XCL1, CCL2, CCL3, CCL4, CCL5, CCL20, CXCL10 or LTB_4 (45-49), or indirectly, by MC mediated upregulation of expression of cell surface adhesion molecules, such as E-selectin, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, on endothelial cells (50-53).

MC also represent sources of mediators that can contribute to the polarization of T cell responses. For example, histamine can promote Th1 cell activation through H1 receptors and suppress both Th1 and Th2 cell activation through H2 receptor (54). Moreover, T cells can

also influence MC development and/or function (44), suggesting the existence of a complex set of cell-cell interactions involving these cell types.

MC lines and certain tissue MC can express CD154, such MC can interact with B cells to induce IgE production in the presence of Il-4 or adenosine but in the absence of T cells (55-58). Moreover, rat MC protease I can enhance the production of IgG1 and IgE by B cells in the presence of IL-4 or LPS (59). Finally, certain MC populations can express mediators that can influence B cell development, such as Il-4, IL5, IL-6 and Il-13 (45, 46, 60, 61). Again, the in vivo relevance of these observations remains largely to be determined. The potential ability of MC to modulate specific antibody responses *in vivo* has been demonstrated by the injection of antigen-pulsed bone marrow-derived cultured MC into naïve mice (62). The antigen-pulsed MC induce a much stronger antigen-specific IgG1 response and more IFN-γ production than do antigen-pulsed B cells or macrophages.

1.1.2.6 Peripheral tolerance

Recent studies have underscored the plasticity of MC in regulating acquired immune responses (63-66), the fact that MC may be instrumental in orchestrating T_{Reg}-cell-mediated peripheral tolerance is unprecedented. It is known that host –derived TGF-β is crucial for the peripheral immunosuppression mediated by T_{reg} cells and it is tempting to speculate that T_{Reg}-cell-activated MC responsible for TGF-β production, or the liberation and activation of TGF-β via other know or unknown factors that MC secrete (67). In addition, TPH1, like indoleamine-pyrrole 2,3-dioxygenase, is an enzyme that can metabolize tryptophan and create a tryptophan-deficient environment (68). As such, this may be a mechanism used by MC to limit T-cell activation.

Table 2: products released by activated mast cells

Class of product	Products
Preformed	Histamine, serotonin (in rodents), heparin and/or chondroitin sulphates, tryptase, chymase, major basic protein, cathepsin, carboxypeptidase-A
Lipid-derived	PGD_2, PGE_2, LTB_4, PAF, LTC4
Cytokines & Growth factors	GM-CSF, IFN-α, IFN-β, IFN-γ,IL-1α, IL-1β, IL-1R antagonist, IL-2, IL-3, IL-5, IL-6, IL-8 (CXCL8), IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17 (IL-25), IL-17F, IL-18, IL-22 (IL-TIF), LIF, LTβ, M-CSF, MIF, SCF, TGF-β1, TNF, TSLP, bFGF, EGF, IGF-1, NGF, PDGF-AA, PDGF-BB, VEGF
Free radicals	Nitirc oxide, superoxide
Others	Corticotropin-releasing factor, urocortin, substance P

Modified from Galli *et al.* (63)

1.1.3 Role of mast cells in pathophysiology

1.1.3.1 Allergic diseases:

MC mediate the “early phase” and “late phase reaction” of type I hypersensisitvity reactions by releasing mediators after crosslinking of surface-bound IgE by allergen in sensitized individuals (69-75). In the late phase reaction, human MC induce the recruitment and local activation of eosinophils by expressing factors such as IL-5 after IgE-dependent activation, as decribed previously for TH2 cells (76), and induce the recruitment of neutrophils by releasing IL-8 and TNF (77, 78).

In vitro studies indicate that human MC also participate in regulating lymphocyte functions in the course of allergic inflammation. After IgE crosslinking, MC produce IL-13, a cytokine that supports the production of allergen-specific IgE by B cells. The release of IL-13

can be further increased by the presence of IL-4, which is known to shift the cytokine profile produced by human MC away from pro-inflammatory cytokines such as TNF, IL-1 and IL-6, to Th2 cytokines including IL-13 (77). Human MC can also regulate T-cell functions, for example through PGD_2, which almost exclusively derives from activated MCand released during allergic reactions (79). Recently, exciting new functions of PDG_2 have been identified that indicate a particular role for PGD_2 at the onset and for the perpetuation of asthma in young adults. The lipid mediator evokes airway hypersensitivity and chemotaxis of T cells, basophils and eosinophils through interaction with two receptors, the prostaglandin D_2 receptor (PTGDR) on granulocytes and smooth muscle cells, and chemoattractant receptor-homologous molecule expressed on Th2 cells (80, 81).

1.1.3.2 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is a chronic, presumably non-infectious, inflammation limited to the large bowel (ulcerative colitis) or anywhere along the gastrointestinal tract (Crohn´s disease); the former is a relatively superficial, ulcerative inflammation, while the latter is a transmural, granulomatous inflammation. The major working hypothesis concerning the pathogenesis of IBD is that the disease is due to abnormal and uncontrolled mucosal immune response to one or more normally occurring gut constituents (82, 83)

Since long time MC were known as a key cell type involved in type I hypersensitivity (84). Since 20 years, this cell type was involved in a lot of non-allergic diseases in internal medicine including chronic obstructive pulmonary disease, Crohn´s disease, ulcerative colitis, etc (Table n°1) (85).

The accumulation of MC at the visible line of demarcation between normal and abnormal mucosa suggested that MC played a crucial role in the pathogenesis of the disease, either causing further damage or limiting the expansion of damage. Nishida *et al* (86), found

that there were greater numbers of MC than macrophages in the lamina propria of patients with inflammatory bowel disease though this was not found in patient with collagenous colitis. Interestingly, increased numbers of MC were observed throughout the lamina propria, particularly in the upper part of lamina propria, whereas increased numbers of macrophages were only seen in the lower part of lamina propria in patients with IBD. This could result from that accumulated MC released their proinflammatory mediators, and these mediators, at least tryptase (87) and chymase (88), induced macrophage accumulation in the lower part of lamina propria.

Not only the number of MC was elevated (89), but also the contents of MC were greatly changed in inflammatory bowel disease in comparison with normal subjects. Laminin, a multi-functional non-collagenous glycoprotein, which is normally found in extracellular matrix was detected in MC in muscularis propria, indicating that MC may be actively involved in the tissue remodeling in Crohn´s diease (90). Similarly, the number of TNF-α positive MC was greater in the muscularis propria of patients with Crohn´s disease than that in normal controls (91). In the submucosa of involved ileal wall of Crohn´s disease, more TNF-α positive MC were found in inflamed area than uninflamed area. Since those TNF-α positive MC were the main cell type that expressed TNFα in ileal wall, the successful treatment of Crohn´s disease with anti-TNF-α antibody could well be the consequence that the antibody neutralized the excessively secreted TNF-α from MC. In chronic ulcerative colitis, increased number of substance P positive MC was observed in gut wall, particularly in mucosa (92), indicating the possibility of neuronal elements being involved in the pathogenesis of the disease.

Lloyd *et al* observed that there were marked degranulation of MC and IgE-containing cells in the bowel wall of patients with Crohn´s disease (93), and this observation later became an important investigation area for understanding the pathogenesis of Crohn´s disease. Dvorak *et al*, described in more detail the degranulation of MC in the ileum of

patients with Crohn´s disease (94) with transmission electron microscopy technique. Similarly, with electron microscopy technique, degranulation of MC was seen in the intestinal biopsies of patients with ulcerative colitis (95). Using immunohistochemistry technique with antibodies specific to human tryptase or chymase, both of which are exclusive antigens of human MC, MC degranulation was found in the mucosa of bowel walls of patients with Crohn´s disease, ulcerative colitis (7) and chronic inflammatory duodenal bowel disorders (96).

MC originated from the resected colon of patients with active CD or UC were able to release more histamine than those from normal colon when being stimulated with an antigen, colon derived murine epithelial cell associated compounds (97). Similarly, cultured colonrectal endoscopic samples from patients with IBD secreted more histamine towards substance P alone or substance P with anti-IgE than the samples from normal control subjects under the same stimulation (98). In a guinea pig model of intestinal inflammation induced by cow´s milk proteins and trinitrobenzenesulfonic acid, both IgE titers and histamine levels were higher than normal control animals (99).

As a proinflammatory mediator, histamine is selectively located in the granules of human MC and basophils and released from these cells upon degranulation. A total of four histamine receptors H1, H2, H3 and H4 have been discovered (100) and the first three of them have been located in human gut (101, 102), proving that there are some specific targets on which histamine can work in intestinal tract. Histamine was found to cause a transient concentration-dependent increase in short-cicuit current, a measure of total ion transport acrossthe epithelial tissue in the gut (103). This could be due to the interaction of histamine with H1-receptors that increased Na and Cl ions secretion from epithelium (104). The finding that H1-receptor antagonist pyrilamine was able to inhibit anti-IgE induced histamine release and ion transport (105) suggests further that histamine is a crucial mediator responsible for diarrhea in IBD and food allergy.

Tryptase is a tetrameric serine proteinase that constitutes some 20 % of the total protein within human MC and is stored almost exclusively in the secretory granules of MC (106) in a catalytically active form (107). The ability of tryptase to induce microvascular leakage in the skin of guinea pig (108), to accumulate inflammatory cells in the peritoneum of mouse (88) and to stimulate release of IL-8 from epithelial cells (109), and the evidence that relatively higher secretion of tryptase has been detected in ulcerative colitis (110) implicated that this mediator is involved in the pathogenesis of intestinal diseases. However, little is known about its actions in IBD but proteinase activated receptor (PAR)-2, a highly expressed receptor in human intestine (111) was recognized as a receptor of human MC tryptase (112). PAR-2 agonists were able to stimulate TNF-α secretion from MC (113) and secreted TNF-α could then enhance PAR-2 expression in a positive feedback manner (114).

Chymase is a serine proteinase exclusively located in the same granules as tryptase and could be released from granules together with other preformed mediators. Large quantity of active form chymase in MC (115) implicates that this MC unique mediator may play a role in MC related diseases. Indeed, chymase has been found to be able to induce microvascular leakage in the skin of guinea pig (116), stimulate inflammatory cell accumulation in peritoneum of mouse (88), and alter epithelial cell monolayer permeability in vitro (117). However, little is know about its actions in IBD but since they are the most abundant granule products of MC and have been demonstrated to possess important actions in inflammation, they should certainly contribute to the occurrence and development of IBD.

1.2 Fibroblasts

1.2.1 Characteristics of fibroblasts

Fibroblasts (FB) are embryologically of mesenchymal origin with a spectrum of phenotic entities ranging from the non-contractile FB to the contractile myofibroblasts (MFB) (Table 3) in a number of intermediate phenotypes having been described (118) including that of the prototypical MFB (119, 120). In addition to the features of active fibroblasts and prototypical MFB are distinguished by the presence of α-smooth muscle actin containing stress fibres, linked in a linear fashion through trans-membrane fibronexus junctions to protruding filamentous fibronectin fibres, increased expression of ED-A fibronectin and gap junctions (118, 119). MFB are further distinguished from smooth muscle cells by their general lack of smooth muscle markers including desmin and smooth musclemyosin. MFB may arise from the transdifferentiation of FB and smooth muscle cells. However, whether MFB-like cells derived from FB and smooth muscle cells from similar or distinct phenotypic populations is debatable and whether FB can differentiate into smooth muscle cells and vice versa is uncertain, although recent studies suggest that FB can differentiate into MFB-like cells with induction of protein expression patterns previously thought to be characteristic of smooth muscle cells (121).

Table 3 Characteristics of the two types of myofibroblasts found in the bowel wall

Intestinal cells of Cajal:	Subepithelial myofibroblast :
-Located in the submucosa and muscularis propria in association with smoothmuscle cells -Thought to be the pacemaker of SMC contraction and bowel movement -From a syncytium	-Display VM (vimentin/myosin) phenotype -Located subepithelial directly beneath the basement membrane -Form a three-dimensional network, „Syncytium“ -Display VA (vimentin/αSMA) phenotype -Thought to be important for epithelial restitution and repair, IEC migration

Modified from Rieder (122)

FB are spindle shaped cells found in the majority of tissues and organs of the body associated with extracellular matrix (ECM) molecules. Characteristic features include expression of vimentin in the absence of desmin and α-smooth muscle actin. When activated, FB exhibit an abundant endoplasmic reticulum and prominent Golgi associated with the synthesis and secretion of ECM molecules including collagens, proteoglycans and fibronectin, as well as, as families of matrix-modifying proteins such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) These latter molecules are important in tissue remodelling and tissue repair (123).

1.2.2 Role of fibroblasts in physiology

1.2.2.1 Maintenance and regulation of extracellular matrix

One of the major functions of FB is the production and homeostatic maintenance of the ECM of the tissue or organ in which they reside. They are metabolically highly active cells, being capable of synthesizing and secreting most ECM components, including collagens, proteoglycans, fibronectin, tenascin, laminin and fibronectin. FB continually synthesise ECM proteins and it has been estimated that each cell can symthesize approximately 3.5 million procollagen molecules/cell/day (124). However, the amount they secrete is regulated by lysosomal enzymes, such as cathepsins B, D and L, with between 10% and 90 % of all procollagen molecules being degraded intracellularly prior to secretion, depending on tissue and age. Regulation of this process appears to provide a mechanism for rapid adaptation of the amount of collagen secreted following injury (125) (Fig 1). In addition, FB produce MMPs and TIMPs, which regulate extracellular degradation of the ECM. FB ECM metabolism is regulated by complex mechanisms including cell-cell and cell-matrix interactions, as well as a multitude of stimulatory and inhibitory mediators, which may be present in their local environment (126).

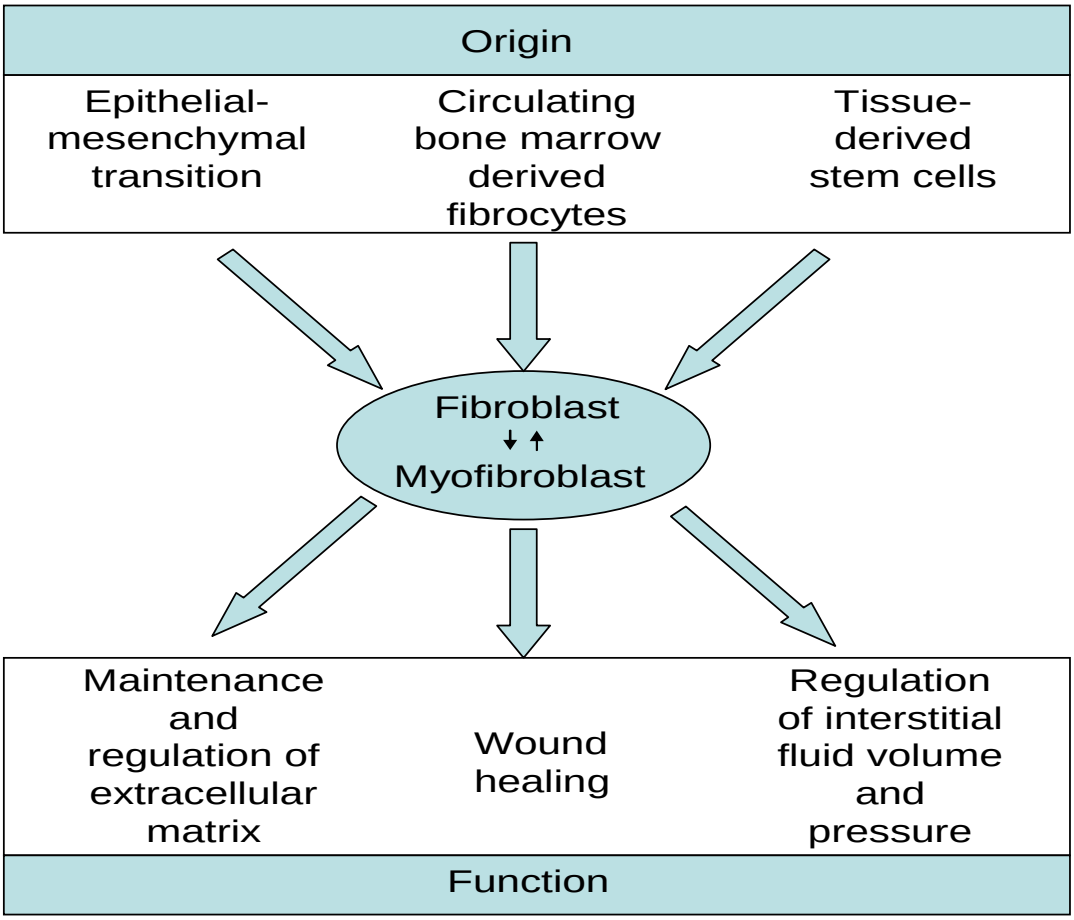


Figure 1: The source and function of fibroblasts in normal states

Modified from McAnulty (127)

Fibroblasts exist as several morphological phenotypes ranging from the extremes of the non-contractile fibroblast to the α-smooth muscle actin stress containing contractile myofibroblasttogether with an intermediate phenotype which has been termed the protomyofibroblasts. Fibroblasts can transdifferentiate into myofibroblasts and there is some evidence to suggest the process may be reversible to at least some extent. Fibroblast populations can be maintained or expanded by proliferation of existing populations, or derived from epithelial-mesenchymal transition, circulating bone marrow-derived fibrocytes or from tissue- derived stem cells. There is also evidence that fibroblasts can undergo mesenchymal-epithelial transition and it has recently been shown that genetic programmes can be induced in fibroblasts to convert them into pluripotent stem cells. Major functions of fibroblasts/myofibroblasts include: synthesis and degradation of the multitude of glycoproteins which make up the specialized extracellular matrices of tissues and organs of the body which contribute to their specific functions, regulation through cell-matrix

interactions of interstitial fluid volume, pressure and appropriate levels of tissue contraction for optimum function; playing a critical role in wound healing through cell-cell and cell-matrix interactions, production and response to mediators, modulation of extracellular matrix metabolism, wound contraction and scar resolution.

1.2.2.2 Regulation of fluid volume and pressure

FB also play an important role in regulating tissue interstitial fluid volume and pressure by interaction of β1 integrin receptors which anchor them to ECM proteins, and particularly the collagen and laminin binding α2β1 integrin via intracellular forces generated through the cytoskeleton (128) (Fig 1). In vitro modeling of these cell-matrix interactions indicate that these processes can be modulated by PDGF and endothelin which enhance contraction and IL-1 and TNF-α which reduce contraction.

1.2.2.3 Wound healing

Following tissue injury FB and MFB play a central role in wound healing and repair (119). The initial processes following injury include clot formation and plated degranulation, releasing mediators to attract inflammatory cells to the wound site which produce additional mediators involved in the recruitment of fibroblastic cells derived from several potential sources. The fibroblastic cells present in this early phase are highly active synthetically replacing the provisional matrix with a more mature ECM including collagens and fibronectin under control for mediators produced by inflammatory cells, injured and regenerating epithelial cells, and FB themselves. As granulation tissue deposition proceed the fibroblasts develop characteristics of myofibroblasts, including the appearance of α-smooth muscle actin containing stress fibres (Fig 1). The appearance of these myofibroblasts correlates with contraction and closure of the wound through focal adhesions between MFB and the extracellular matrix. During the final phases of remodeling and resolution the production of MMPs and TIMPs by cells including FB changes from a balance favouring ECM deposition

to a matrix degrading environment and MFB are removed by apoptosis.

1.2.2.4 Products released by activated fibroblasts

FB and MFB are positive for the production of SCF, Granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-6 and Transforming growth factor beta (TGF-β) (129).

SCF also named mast cell growth factor or kit-ligand, has only recently been cloned and has been shown to be encoded on human chromosome 12. It may be of specific importance in physiology and pathology since it is produced by several cell types (e.g. fibroblasts, keratinocytes, endothelial cells) and since it affects MC growth, survival, secretion and adhesion as well as migration into tissues.(130)

GM-CSF is a naturally occurring substance that is made by the body in response to infection or inflammation. It acts on the bone marrow to increase the number of two types of white blood cells that fight infection, granulocytes (neutrophils) and monocytes, and makes them more effective.(131)

IL-1 β is a pro-inflammatory cytokine and a potent endogenous inhibitor of gastric acid secretion. IL-1β is a soluble protein which are involved in the activation of T-lymphocytes and B-lymphocytes.(132)

IL-6 is a pro-inflammatory cytokine secreted to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. In terms of host response to a foreign pathogen, IL-6 has been shown, in mice, to be required for resistance against the bacterium, *Streptococcus pneumoniae* (133).Furthermore, IL-6 is known to induce survival of mouse MC.Indeed, Hu, ZQ et al (134) have shown that IL-6 induced MC development from spleen cells of mouse whereas IL-1, IL-5, GM-CSF, TGF-β, and even the MC growth factors, IL-9 and SCF, failed to do so.

TGF-β is a multifunctional peptide that controls proliferation, differentiation, and other

functions in many cell types. TGF-β acts synergistically with TGF-α in inducing cellular transformation (MIM 190170). It also acts as a negative autocrine growth factor. Specific receptors for TGF-β activation trigger apoptosis when activated. Many cells synthesize TGF-β and almost all of them have specific receptors for this peptide (135).

1.2.3 Role of fibroblasts in pathophysiology

1.2.3.1 Inferred from fibroblasts in the diseases

A lot of disease associated with diminished or excess deposition of ECM are likely to be related to dysregulation of the injury repair response and fibroblast function. In this context " Injury" is broad ranging including environmental, infectious, cancerous, traumatic/mechanical, autoimmune and drug-induced insults. Thus, diseases in which fibroblasts, in their various phenotypic guises, play a central role may affect almost all tissues and organs of the body (Fig 2). Their importance is further highlighted by the suggestion that almost half of all deaths are associated with fibrosing conditions. Diseases associated with either increased or decreased ECM deposition, or contraction of tissues result in distorted tissue architecture, impaired function and in many cases, particularly where the vital organs are involved, significant morbidity and mortality. Dysregulation of several phases of the injury repair response, including chronic or repetitive injury, an inappropriate inflammatory response, an altered balance of ECM metabolism and deposition, altered phenotypic profiles or persistence of myofibroblasts contribute to aberrant tissue repair.

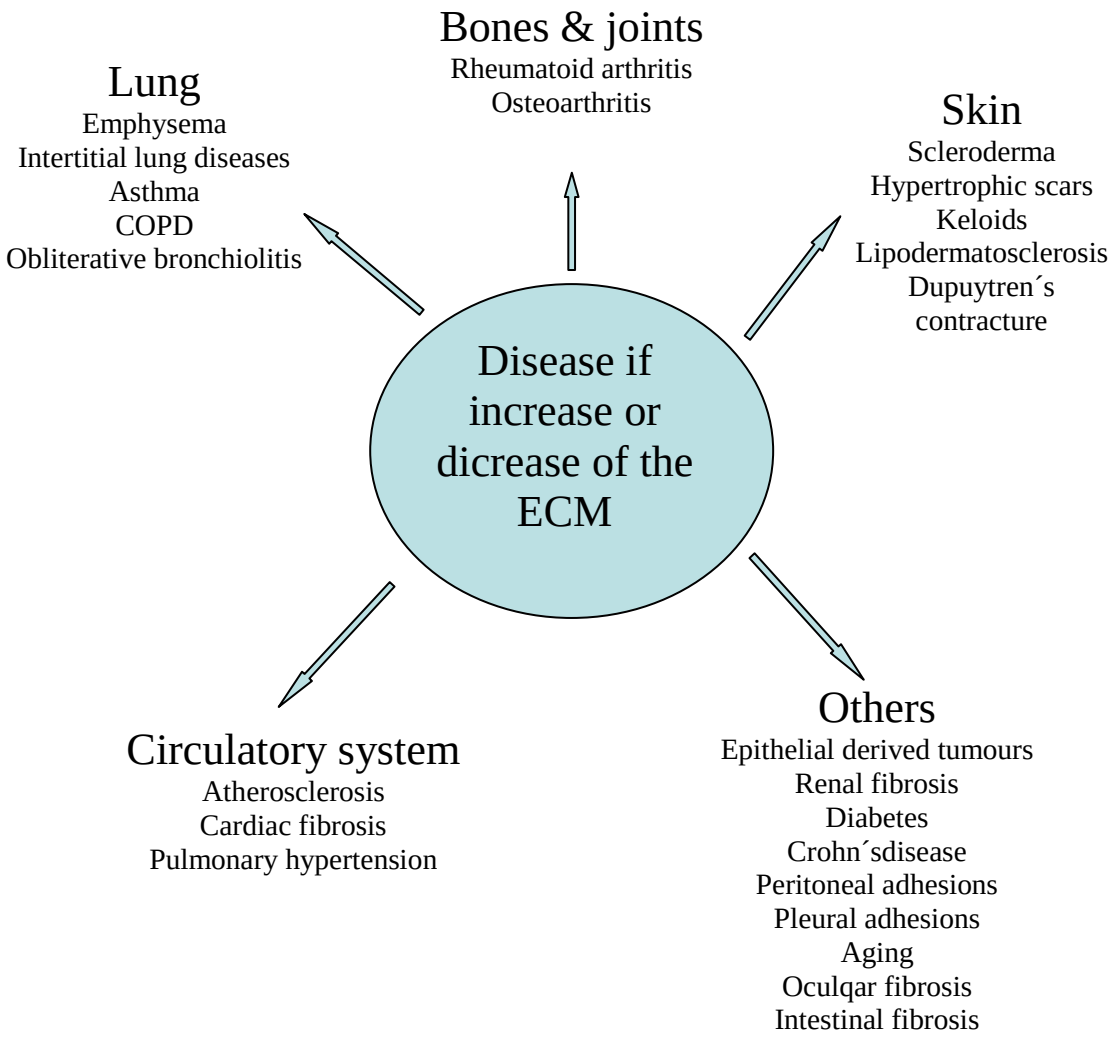


Figure 2: Fibroblasts in pathological states

Modified from McAnulty (127)

Dysregulated or inappropriate fibroblast function is associated with pathologies which diminished or excess extracellular matrix deposition, orinappropiate tissuecontraction is feature. Such conditions affect almost all tissues and organs of the body.

1.2.3.2 MMPs and inflammatory bowel disease

FB and MFB produce the MMPs. MMPs are increasingly recognized to play a physiological role in intestinal homeostasis as well as a pathogenetic role in the initiation and perpetuation of intestinal inflammatory response (135). Accumulating data demonstrate that some of the MMPs (MMP-2, MMP-3, MMP-7) are constitutively expressed and regulate

physiologic processes such as barrier function and mucosal defense, while others (MMP-1, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13) are undetectable in normal intestine but their dysregulated expression during inflammation may play a role in cell adhesion, immune cell migration, and impaired wound healing. Although much work needs to be done on the precise role and regulation of MMPs, it is evident that the final outcome of inflammatory response depends on a balance between anti-inflammatory MMPs, proinflammatory MMPs and TIMPs.

1.2.3.3 Fibrosis and Crohn´s disease

Inflammation is associated with an infiltrate of immune cells, such as T cells, macrophages and neutrophils, and it also often causes severe damage to the tissue in which it occurs. In the case of intestinal mucosa, severe inflammation is followed by a loss of epithelial cells and a degradation of ECM in the lamina propria, clinically leading to ulcerations. Enzymes and mediators mainly secreted by monocytes, intestinal macrophages and granulocytes are responsible for this tissue damage. This continous inflammation and tissue degradation may consequently lead to fibrosis and stricture formation.

Oxidants are important contributors to mucosal, and eventually submucosal, tissue destruction. Oxygen metabolites, such as oxygen or hydroxide radicals, are produced in large amounts by infiltrating leucocytes in the inflamed mucosa (136). The normal intestinal wall contains relatively small amounts of antioxidative enzymes (137).

Besides radical formation, infiltrating and locally activated immune cells respond to intestinal inflammation by secreting ECM-modifying and ECM-degrading enzymes (138). This permits furtherinfiltration of immune and non-immune cells into the inflamed area, finally paving the way for the migration of myofibroblasts.

If the defect is deeper, with subepithelial tissue damage, the area below the basement membrane has to be reconstituted in addition to the epithelial surface. One of the key events

in that process is the contraction of the underlying lamina propria to limit the wound area the epithelium finally has to cover. A rapid wound closure is important to reduce the time of impaired barrier function of the intestinal wall. Recent studies have provided evidence for the deleterious consequences of an uncontrolled and longlasting translocation of bacteria from the gut lumen into the mucosal wall (139). It is crucial to prevent bacterial translocation, or-if impossible-to rapidly detect and sense translocated bacteria, a lesson we have learnt from the first susceptibility gene for CD, NOD2/CARD15 (139). In fact, variants of NOD2/CARD15 causing an increased risk of developing CD are also associated with a higher frequency of fibrosing and structuring disease (140-145). Further clinical evidence suggests a genetically determinated risk to develop strictures. Some patients obviously have rapidly recurring strictures, whereas others permanently have an inflammatory, non-stricturing diease type (Fig. 3).

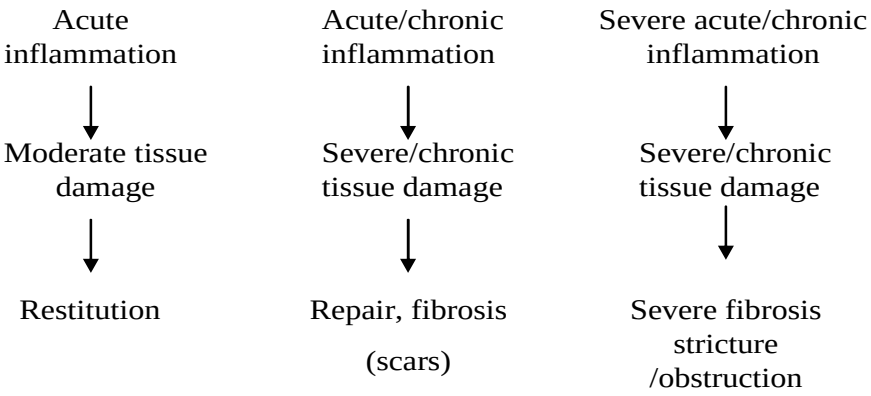


Figure 3: Severity of inflammation and tissue repair

From Rieder(122)

Acute intestinal inflammation is normally followed by moderate or limited tissue damage and complete restitution. More severe acute or moderate chronic inflammation may result in severe or chronic tissue degradation and damage, followed by repair, and may also be accompanied by fibrosis and scars. However, severe acute and longlasting chronic tissue damage may be associated with severe fibrosis, leading to intestinal strictures and obstruction.

1.3 Aim of my doctoral thesis

Garbuzenko *et al* (146) have shown that the human MC line HMC-1 modulate proliferation, collagen synthesis, and collagenase activity of lung fibroblast cell line FHS 738 (no. HTB-157). MC interact with lung fibroblasts both directly and in a synergistic manner with bronchoalveolar (BAL) macrophages and thereby may play an important role in the modulation of fibrosis in the lung. Kendall *et al* (147) have shown that IgE-dependent activation of mouse MC can result in the release of mediators that promote fibroblast proliferation in the absence of any other cell type and suggest that mast cell-derived TNF-alpha and TGF-beta 1 contribute substantially to this effect. The role of primary human MC on primary human FB and vice versa is largely unknown because of the difficullty to obtain the cells from human tissue. The group of Prof. Bischoff found out that FB suppress apoptosis in human intestinal MC independently of stem cell factor, IL-3, IL-4 and nerve growth factor. But the factor was still unidentified. Effects of human intestinal MC on human intestinal FB were also still unknown.

Thus, the aim of the study was to characterize the interaction of primary human intestinal MC and primary human intestinal FB in a co-culture model to elucidate some of the underlying molecular mechanisms of the cross-talk between MC and FB.

Materials and Methods

2.1 Reagents

Commercial reagents were obtained from the following sources : HEPES, D-glucose, gelatine type B, chymopapain, acetylcysteine, and trypan blue from Sigma Chemical Co (St Louis, MO); ampicillin from Bayer AG (Leverkusen, Germany) ; RPMI 1640 medium, penicillin, streptomycin, L-glutamine, FCS, gentamycin, and PBS ($MgCl_2$/$CaCl_2$) from LifeTechnologies (Grand Island, NY); Turk´s staining solution from Fluka AG (Buchs, Switzerland); metronidazol from Fresenius (Bad Homburg, Germany); DNase, BSA (fraction IV), pronase, collagenase D, and elastase from Boehringer Mannheim (Mannheim, Germany); and Percoll from Pharmacia Biotech (Freiburg, Germany).

2.2 Buffers

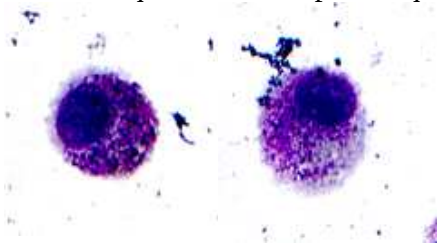
For cell preparation, buffers were used as described previously (149, 150). Cells were cultured in RPMI 1640 without phenol red supplemented with10% (v/v) heat-inactivated FCS, 25mM HEPES, 2mM L-glutamine, 100 µg/ml gentamicin, 100U/ml penicillin, and 100µg/ml streptomycin. For cell stimulation, cells were resuspended in HEPES/albumin (HA) buffer (containing 20mM HEPES, 0.25 mg/ml BSA, 125mM NaCl, 5mM KCL, and 0.5 mM glucose) supplemented with 1mM $CaCl_2$ and 1 mM $MgCl_2$. All buffers were sterilized using 0.22 µm bottle top filters (Falcon, Heidelberg, Germany).

2.3 Isolation, purification, and culture of human intestinal mast cells

MC were isolated under sterile conditions in a lamina air flow from surgical tissue specimens (macroscopically normal tissue) using a four-step enzymatic dispersion method that was previously described (77, 151-154). Briefly, macroscopically normal human intestinal tissue was obtained from surgical specimens (border sections, free of tumours cells

as determined by histologic examination of the tissue). The tissue was placed in 4°c cold buffer immediately after resection until dissection was started. The mucosa was separated mechanically from the submucosa/muscular layers. Mucus was removed by incubation with acetylcysteine at 1 mg/ml, and epithelial cells were detached by EDTA at 5 mM. The tissue was enzymatically digested by a four-step incubation (each for 30min) with four enzymes (3mg/ml pronase corresponding to 21 U/ml, 0.75 mg/ml chymopapain corresponding to 0.39 U/ml, 1.5 mg/ml collagenase D corresponding to 0.405 U/ml, and 0.15 mg/ml elastase corresponding to 15.75 U/ml). During the first digestion step, the mucosa was chopped finely with scissors. For the third and fourth digestion steps, the incubation buffer was supplemented with DNase at 15µg/ml (Corresponding to 15 U/ml). The cells freed after the last two digestion steps were separated from tissue fragments by filtration through a polyamide Nybolt filter (Pore size, 300µm; Swiss Silk Bolting Cloth Manufacturing Co. Ltd, Zurich, Switzerland), washed, pooled, and counted after staining with Turk´s solution. The viability of cells was measured by dye exclusion using trypan blue staining. Twenty-nine percent (median) of the cells stained positive (range, 20-41%); the positive cells were mostly epithelial cells and other cell types, but rarely MC. A differential count of cytocentrifuge smears stained with May-Grunwald-Giensa (Sigma) revealed an MC percentage of 4.6 ± 2.1%. After overnight culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM glutamine, 100 µg/ml streptomycin, 100 µg/ml gentamycin, 100 U/ml penicillin, and 0.5 µg/ml amphotericin; all from Invitrogen (Paisley, U.K), MC were enriched from non-adherent cells by positive selection of c-*kit*-expressing cells using magnetic cell separation (MACS system; Miltenyi Biotec, Bergisch-Gladbach, Germany) coupled with the anti-c-*kit* mAb YB.B8 (5 ng/ml; BD PharMingen, Hambourg, Germany) (76, 151-154). Cells were resuspended in 1 ml of HA buffer containing 1 mg/ml albumin and 5 µg/ml mAb YB5.B8 (directed against human c-kit) and incubated for 30min at 4°c with gentle rolling as preiously described (155). The cells were then washed in HA buffer and

resuspended in 500 µl of HA buffer containing1 mg/ml albumin. Finally, the cells suspension was incubated with a goat anti-mouse IgG Ab coupled to paramagnetic beads (Miltenyi Bioted, Bergisch Gladbach, Germany) for 30 min at 4°C. During incubation the tubes were gently rolled. After washing in HA buffer, MC were enriched bymagnetic separation of the cells using a MACS C1 column placed in a magnetic field (Miltenyi Biotec). The fraction containing the c-kit-positive cells (MC purity 50-90%) was cultured (1-2 x 10^5 MC/ml) in presence of recombinant SCF (rSCF; 25 ng/ml; Peprotech, Rocky Hill, NJ). In such conditions, MC purity is increased up to 97-100% after 2-4 weeks. Purification of MC can also be obtained by long-term culture of non-enriched cell faction (156). This approach has the advantage of higher cell numbers and a better culture sufficiency. The disadvantages are that the purity is often poor and the required culture period quite long.

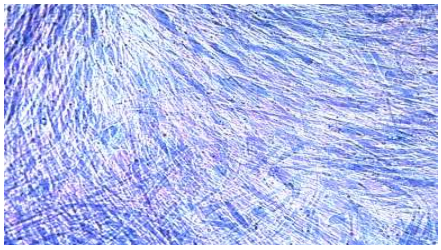


MC after isolation, purification and culture with SCF (staining in May-Grünwald/Giensa)

2.4 Isolation, purification, and culture of human intestinal fibroblasts

Human intestinal FB were obtained from the adherent cell fraction isolated from surgical specimens (Figure 3). Cells were maintained in culture medium for 1-2 weeks until they formed a subconfluent layer. For subculturing, cells were detached by trypsin/EDTA treatment for 5-20 min (0.05%/0.02%; Biochrom, Berlin, Germany).Then, the cells were seeded into fresh dishes at a density of 1 x 10^5 cells/ml. Such passages were repeated two to four times. Depending on the cellular confluence, the culture medium was exchanged every 3-4 days. To assess FB purity, cells were analysed by immunocytochemistry directed towards vimentin, cytokeratin and anti-smooth muscle actin. Vimentin was expressed in all cells confirming their mesenchymal origin (157). The epithelial cell marker cytokeratin was

generally not expressed, with the exception of two of seven analysed FB cultures displaying, respectively 4 and 9.5% positive cells. In three of seven FB preparations, we found 19-42% anti-smooth muscle actin-positive cells indicating the presence of myofibroblasts in these cultures. FB preparations were negative for CD31 and von Willebrand factor as assessed by flow cytometry and thus were not contaminated by endothelial cell (20 and data not shown).



Confluent FB after isolation, purification and culture *(staining in May-Grünwald/Giensa)*

2.5 Co-culture of mast cells and fibroblasts

The principal experimental plan of all experiments is summarized in Fig. 4. For MC/FB co-culture or MC culture in the presence of FB-conditioned medium precultured 97-100% pure MC were used. Before using MC, cultured cells were washed twice to remove rSCF. For co-culture, $1x10^5$ MC were seeded onto confluent FB monolayers in 24-well plates (in Nalge Nunc International, Roskide, Denmark) with or without separation of the two cell types using Transwell membranes (0.2-µm pore size; Nalge Nunc International). For further evaluation of MC/FB interactions, MC were cultured in the presence of FB-conditioned medium (FB supernatants). FB supernatants were harvested from confluent FB monolayers cultured for 24h in 3 ml of culture medium in 25-cm^2 culture flasks with or without supplementation of Il-1β (10ng/ml) or TNF-α (10ng/ml). FB-conditioned medium was stored at-80°C. MC recovery rates were expressed as a percent of cell numbers at start of the culture.

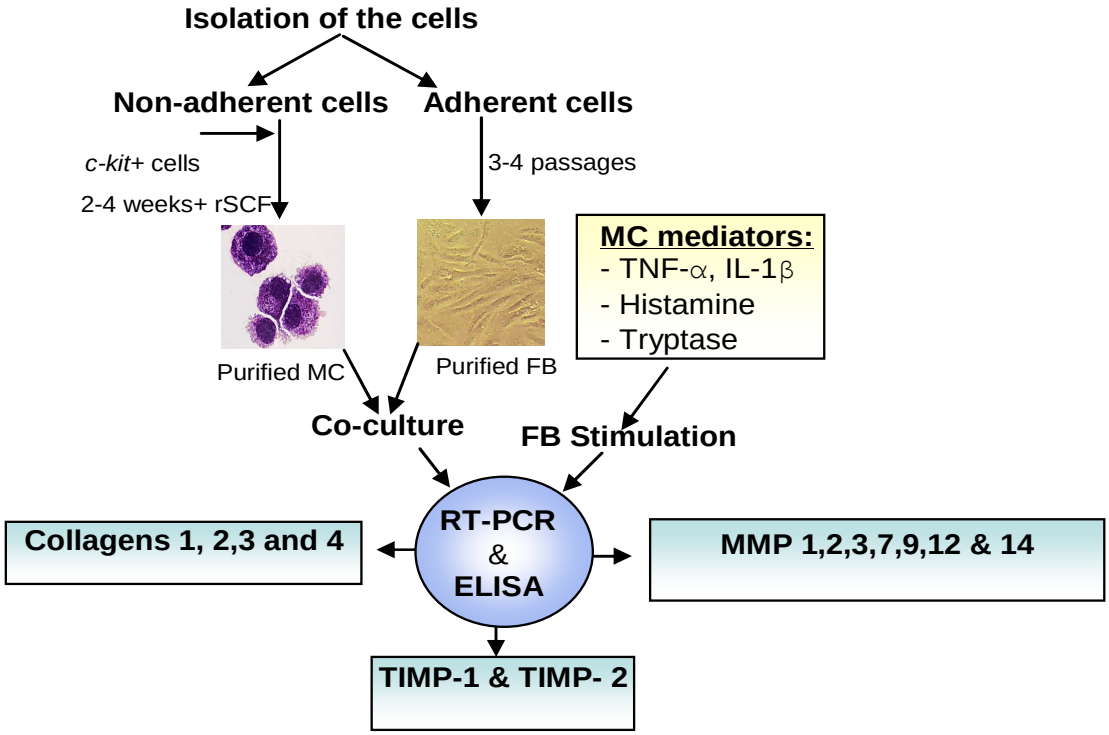


Figure 4: Experimental setup

2.6 RNA isolation and RT-PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) (76). For RT-PCR, 200 ng of total RNA was treated for 15 min at 37°C with 1U RNase-free DNase (Promega, Madison, WI) to remove genomic DNA. After denaturation for 10min at 70°c, cDNA was synthesized for 1h at 37°C by adding Superscript™ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 20 pmol oligo dT primers (Pharmacia Uppsala, Sweden). 1/10 vol of the cDNA was used for one PCR reaction. Thirty-five cycles (60s at 94°C, 80s at 60°c, 70s at 72°C) were performed with 2,5U Taq DNA polymerase (Invitrogen) and 20pmol of the primers (synthesized by MWG) for quantitative real time PCR assays, specific sense and antisense primers for cDNAs were used (158-160).

Table 4: Primers sequences

	Sens	Antisens
MMP-1	5´-CAGTGGTGATGTTCAGCTAGCTCA-3´	5´-GCCGATGGGCTGGACA-3´
MMP-2	5´-GAGGACTACGACCGCACAA-3´	5´- CTTCACTTTCCTGGGCAACAA-3´
MMP-3	5´-GTTCCGCCTGTCTCAAGATGA-3´	5´-TACCCACGGAACCTGTCCC-3´
MMP-7	5´-GGATGGTAGCAGTCTAGGGATTAACT-3´	5´-GGAATGTCCCATACCCAAAGAA-3´
MMP-9	5´-CAAGCTGGACTCGGTCTTTGA -3´	5´-ACCGACGCGCCTGTGTAC-3´
MMP-12	5´-CGCCTCTCTGCTGATGACATAC-3´	5´-CAGGATTTGGCAAGCGTTG-3´
MMP-14	5´-GAACTTTGACACCGTGGCCAT-3´	5´-CCGTCCATCACTTGGTTATTCCT-3´
TIMP-1	5´-TGTTGTTGCTGTGGCTGATAGC-3´	5´-AAGTTCGTGGGGACACCAGA-3´
TIMP-2	5´-AGTGGAACGCGTGGCCTAT-3´	5´-CGGGAGACGAATGAAAGCA-3´
Collagen a1 (I)	5´-TCCGGCTCCTGCTCCTCTTA-3´;	5´-GTATGCAGCTGACTTCAGGGATGT-3´
Collagen a1 (III)	5´-AATGGTGGCTTTCAGTTCAGCT-3´	5´-TGTAATGTTCTGGGAGGCCC-3´
Collagen a1 (IV)	5´-ATGCCCCCTGCCCATT-3´	5´-ACAGGCCAATCCAAGGTTAGAG-3´
GAPDH	5´-ACCACAGTCCATGCCATCAC-3´	5´-TCCACCACCCTGTTGCTGTA-3´

2.7 Measurement of IL-6 and MMP-1 in supernatants

IL-6 and MMP-1 were quantified by ELISA according to the manufacturer's instructions. The ELISA kit from R&D Systems (Minneapolis, MN) was used to measure IL-6, and the ELISA kit from Amersham Pharmacia Biotech (Piscataway, NJ) to measure MMP-1.

2.8 Stimulation of mast cells and inhibition of fibroblasts

Mast cells were stimulated in culture medium without addition of SCF by FcεRI cross-linking using the purified mAb 22E7 (provided by Hoffmann-La Roche, Nutley, NJ) directed against a non-IgE binding epitope of the FcεRI α chain.

FB were treated with the inhibitors cyclosporin A (Novartis, Basel, Switzerland), actinomycin D, apigenin, PD98059, Gö6976, and wortmannin (all from Calbiochem, La Jolla, CA) for 1 h prior to stimulation at the concentrations indicated (157). Substances were

dissolved in H_2O, ethanol, or dimethyl sulfoxide. Controls were carried out with ethanol and dimethyl sulfoxide at a concentration equivalent to the lowest dilution of the inhibitor (≤0.1%).

2.9 Analysis of MMP-1 in human gut sections by means of immunohistochemistry

Serial 4 µm biopsy sections were deparaffinized in xylene, step-rehydrated through graded alcohol, and washed with Tris-buffered saline (TBS, 20 mMTrizma base, 150 mM NaCl, pH 7,6). Sections were then incubated for 90 min with either MMP-1 antibody (1:1000) (abcam ab8480, Cambrige, UK). Sections were washed in TBS, and incubated at room temperature for 30 min with an appropriate biotinylated anti-mouse (1:200) (Amersham, Les Ulis, France), then for 30 min with an extravidin-alkaline phosphataseconjugate (1:200) (Sigma). Finally, sections were incubated in Fast Red TR/Naphtol AS-MX (Sigma), and allowed to develop for 20 min at room temperature.Sections were counterstained with Mayer´s haematoxylin(Fluka, Saint Quentin Fallavier, France), and mounted in gelatin-glycerol (1:1)

2.10 Statistics

All data are expressed as mean ± SEM if not indicated otherwise. Significance was assessed by using the Wilcoxon test. A value of $p < 0.05$ was considered to be statistically significant.

Results

3.1 Fibroblast derived IL-6 supports intestinal mast cell survival

Sellge G et al (1) have shown previously that human intestinal FB suppress apoptosis in human intestinal MC independent of SCF, IL-3, IL-4 and NGF(1). We hypothesized that IL-6 could be considered as the responsible factor because of its ability to trigger mast cell development from progenitor cells (134, 161-163). As shown in Fig. 5, intestinal FB are capable of producing IL-6 provided that they were stimulated directly by MC in co-culture, and less efficiently so by MC mediators such as TNF, IL-1β, tryptase or histamine.

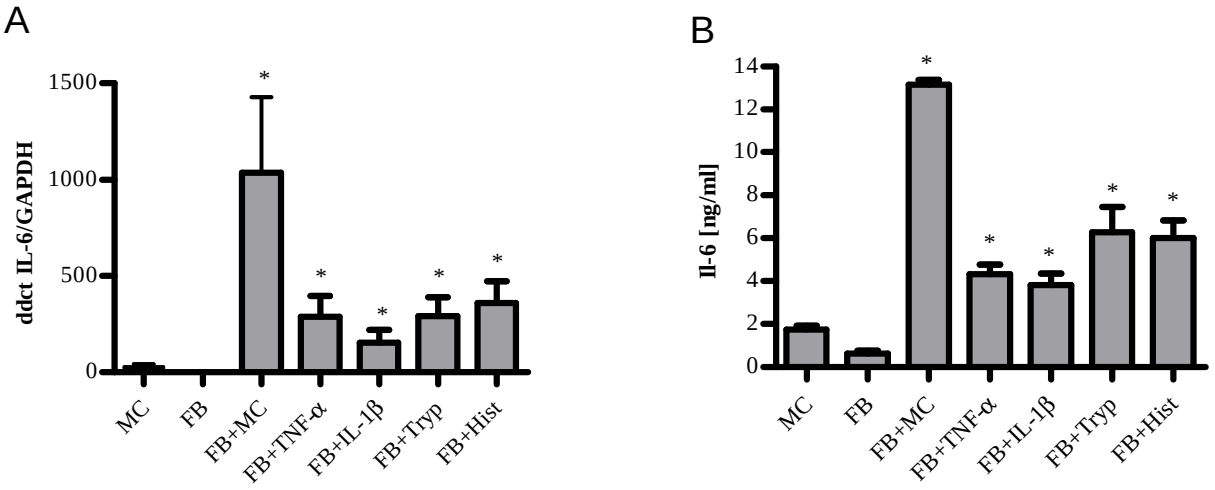


Figure 5: **MC stimulate FB to express IL-6**.

MC and FB were cultured alone, or co-cultured by seeding MC onto confluent FB layers (FB+MC), or FB were incubated with the MC mediators TNF, IL-1β, tryptase, and histamine (each 10ng/ml) for 24h. ***A.*** *IL-6 mRNA expression was analyzed by real time RT-PCR.* ***B.*** *IL-6 protein produced by FB was measured by ELISA. Data are shown as means ± SEM (n=6, * p<0.05 compared to FB alone).*

In order to investigate the role of IL-6 for the survival of intestinal MC, MC were incubated with different concentrations of IL-6 (Fig. 6A). Low nanogram amounts of IL-6 supported MC survival up to 2 weeks in culture in a concentration dependent manner. In contrast to treatment with SCF, all MC incubated with IL-6 died after 3 weeks of culture (Fig. 6A).

Interestingly, culture of MC with supernatants from FB stimulated with TNF-α or IL-1β had similar effects as IL-6. MC survived for two weeks in culture, but not longer (Fig. 6 B). Furthermore, MC survival for two weeks of culture with FB supernatant could be blocked using an anti-IL-6 Ab (Fig. 6C). To exclude a possible toxicity of the neutralizing Ab to IL-6, MC were incubated in the presence of SCF with or without anti-IL-6 Ab. No differences were observed between MC cultured with SCF and MC cultured with SCF and anti-IL-6 Ab (Fig 6D). Taken together, these findings suggest that MC factors trigger FB to produce IL-6, and, vice versa, that IL-6 supports MC survival.

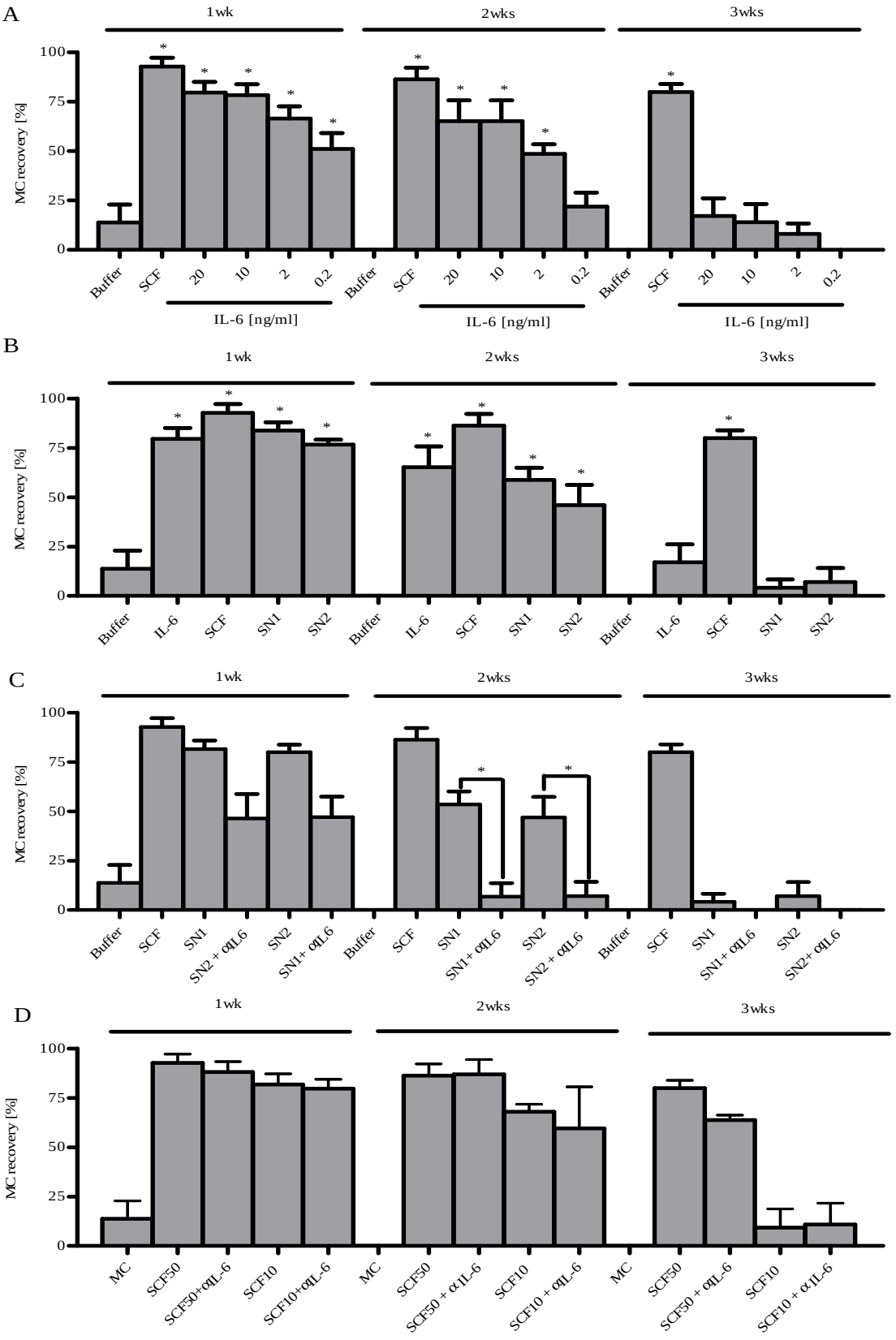


Figure 6: **FB-derived IL-6 supports intestinal MC survival.**

MC survival is expressed as percentage of recovery of initial MC numbers at the starting

*point of the experiments. MC recovery was assessed after 1, 2, and 3 weeks (n=8, * p<0.05 compared to MC cultures without additions, means ± SEM are shown).* ***A****. MC were incubated with different concentration of IL-6 (0.2, 2, 10, 20ng/ml) or 25 ng/ml SCF (positive control).* ***B****. MC were stimulated with 20 ng/ml IL-6, or 25 ng/ml SCF, or supernatants derived from FB that have been stimulated with TNF (=SN1) or IL-1β (=SN2) for 24 h.* ***C****. MC were incubated with 25 ng/ml SCF compared to SN1 and SN2 with or without addition of an blocking antibody directed against IL-6.* ***D****. Blocking antibody direct against IL-6 were not cytotoxic for the MC.*

3.2 Human intestinal mast cells induce MMP-1 synthesis in fibroblasts

To further analyze the cross-talk between intestinal MC and intestinal FB with respect to a potential role in the pathogenesis of fibrosis, confluent human intestinal fibroblasts were co-cultured with MC and analyzed for the expression of collagens and MMPs. Non adherent MC were separated from adherent FB by several washing steps before FB mRNA expression was quantified by real time PCR. Transcripts for MMP-1, 2, 3, 7, 9, 13 , and 14 (Figure 7A), TIMP-1 and 2 (Figure 7B) as well as for collagen a1(I), a1(III) and a1(IV) (Figure 7C) were measured (n=6). Only the expression of MMP-1 was significantly increased in response to the direct cell-cell contact of FB with MC.

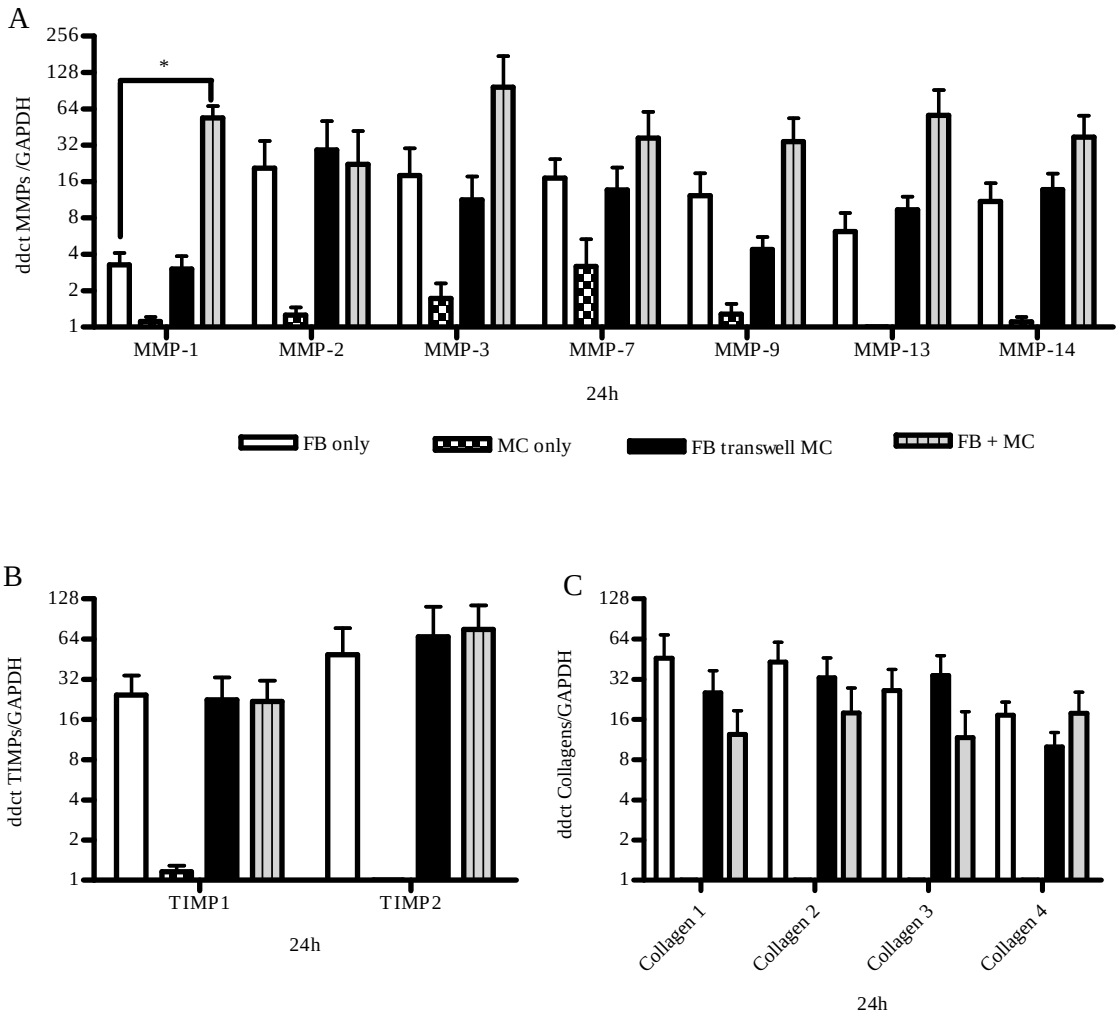


Figure 7: Co-culture between MC and FB induce MMP-1 synthesis.

FB were co-cultured with MC for 24h and mRNA expression was analyzed by real time RT-PCR. ***A****, MMP family members (n=6, * p<0,05 compared to FB alone).* ***B****, TIMP-1 and 2, and* ***C****, Collagens.*

To further evaluate the role of MC in the induction of MMP-1 expression in FB, confluent FB were challenged with increasing numbers of MC. As shown in Fig. 8A and B, MMP-1 expression was clearly dependent on MC numbers. Time course experiments showed a substantial induction of MMP-1 mRNA and protein after 24h of co-culture (Fig. 8C and D).

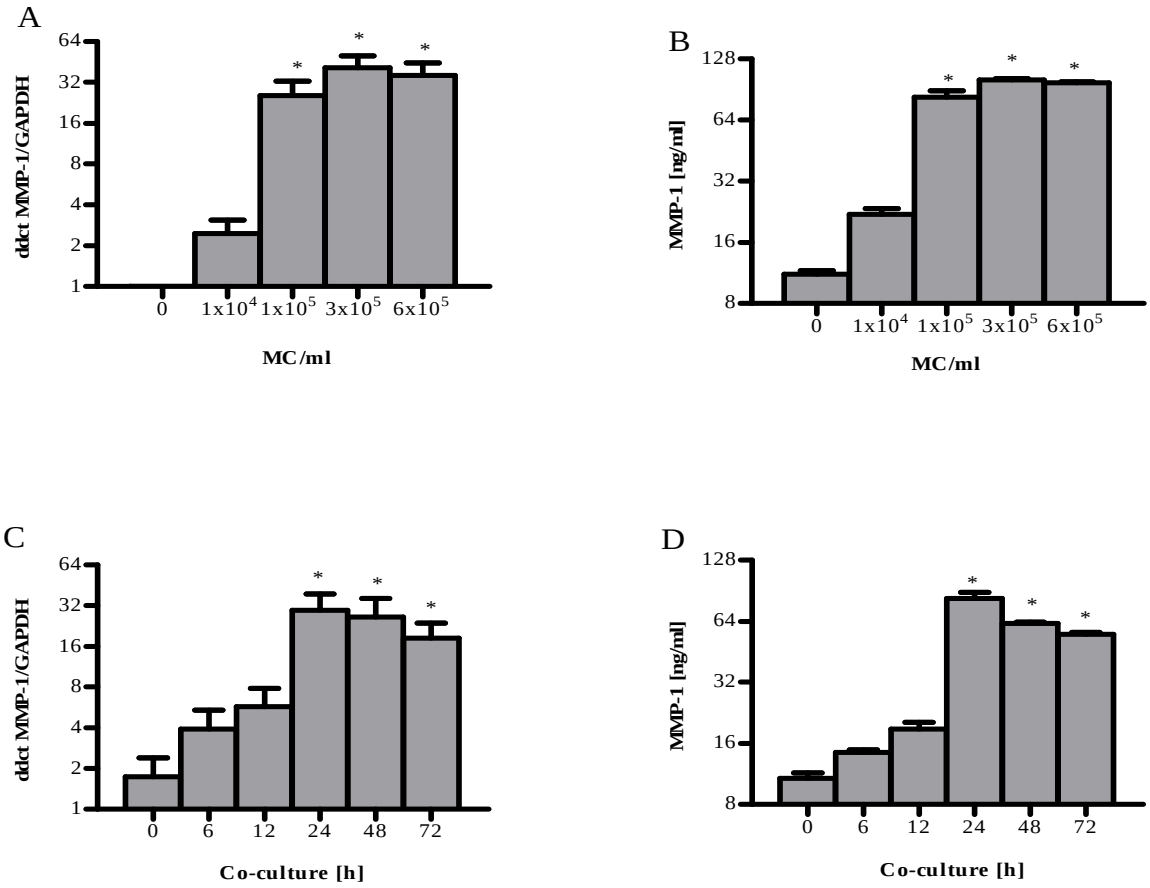


Figure 8: **Human intestinal MC induce MMP-1 synthesis in FB.**

A, B. *FB were co-cultured with different numbers of MC (1 x 10^4 – 6 x 10^5 MC/ml) for 24 h (n=6, * p<0.05 compared to FB alone, means ± SEM).* ***A.*** *MMP-1 mRNA expression was analyzed by real time RT-PCR.* ***B.*** *MMP-1 protein produced by FB was measured by ELISA.* ***C, D.*** *FB were co-cultured with 1 x10^5 MC/ml for 6-72 h (n=6, * p<0.05 compared to FB alone, means ± SEM).* ***C.*** *MMP-1 mRNA expression was analyzed by real time RT-PCR.* ***D.*** *MMP-1 protein produced by FB was measured by ELISA.*

3.3 Role of mast cell mediators in the induction of MMP-1

FB or MC alone, unstimulated or stimulated by 100 ng/ml mAb 22E7 causing IgE receptor (FcεRI) cross-linking, failed to express MMP-1 mRNA or protein. The same result was obtained when confluent FB were co-cultured with MC separated by a transwell membrane. However, if MC were stimulated by FcεRI crosslinking for one hour, MMP-1 expression was significantly increased in FB even though they were separated from MC by a transwell membrane (n=6, p=0.03) (Fig. 9 A and B). Interestingly, MMP-1 expression in FB

could not be further enhanced by MC stimulation if MC and FB were co-cultured in direct cell-cell contact (Fig. 10 A and B). In contrast, TIMP-1, the specific inhibitor of MMP-1, was not induced in FB by either stimulated or unstimulated MC (data not shown). The next step was to determine which MC mediators were responsible for the induction of MMP-1 expression in FB. FB were treated with several MC mediators for 24h. As shown in Fig. 10 C and D, TNF, IL-1β, tryptase and histamine induced a significant increase of MMP-1 expression in FB. TNF induced MMP-1 expression significantly more strongly compared to histamine and tryptase. To demonstrate the effect of MC derived TNF or IL-1β, blocking Abs were used to inhibit TNF or IL-1β in FB-MC co-culture experiments. As expected, inhibition of TNF or IL-1β resulted in a significant decrease of the induced MMP-1 expression in FB (Fig. 9 E and F).

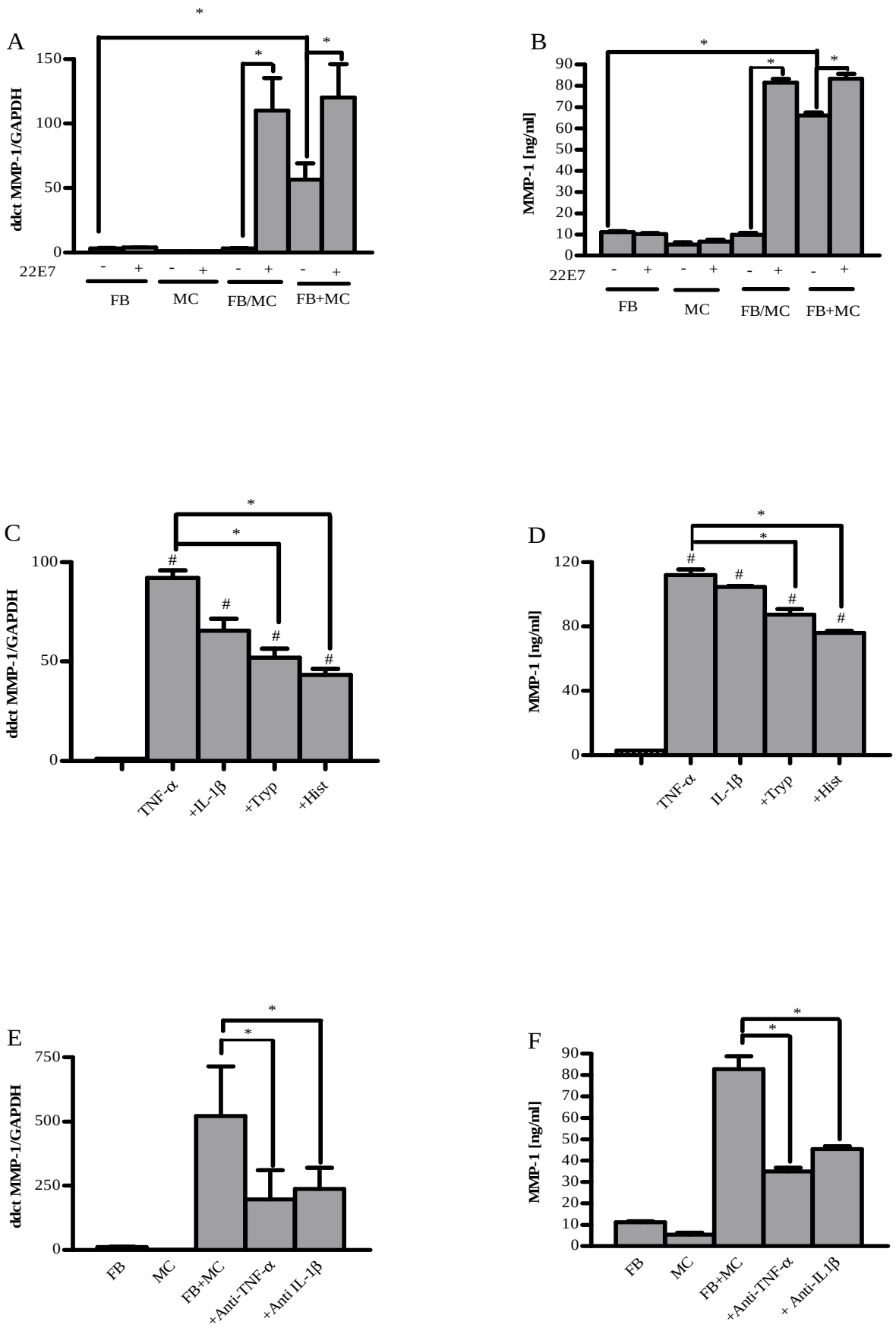


Figure 9: **Role of MC mediators in the induction of MMP-1. A, B.**

FB were cultured in the absence of MC (FB), or co-cultured with MC by seeding MC onto

confluent FB layers (FB+MC), either with or without addition of the antibody 22E7 causing MC activation by FcεRI crosslinking. ***A.*** *MMP-1 mRNA expression was analyzed by real time RT-PCR.* ***B.*** *MMP-1 protein was measured by ELISA (n=6, * p<0.05, means ± SEM).* ***C, D****. FB were incubated with the MC mediators IL-1β, TNF, tryptase, and histamine (each at 10 ng/ml) for 24h.* ***C.*** *MMP-1 mRNA expression was analyzed by real time RT-PCR.* ***D.*** *MMP-1 protein produced by FB was measured by ELISA (n=6, * p<0.05, # p<0.05 compared to FB alone, means ± SEM).* ***E, F.*** *MC and FB were cultured alone or FB were co-cultured with MC, with or without addition of blocking antibodies directed against TNF or IL-1β, respectively (n=6,* p<0.05, means ± SEM).*

3.4 MMP-1 induction is blocked by the MEK inhibitor PD98059

FB were treated with several inhibitors prior to co-culture with MC to analyze signalling pathways involved in the induction of MMP-1 expression. Following treatment of FB with the immunosuppressive drug cyclosporin A, known to prevent activation of the transcription factor NF-AT (164)**,** MMP-1 mRNA transcription and protein expression was not reduced. The same was found after treatment of FB with Gö 6976, an inhibitor of PKC**,** or with wortmannin, a specific inhibitor of PI3-K (165)**,** suggesting that NF-AT, PKC, or PI3K are not involved in MMP-1 induction (data not shown). In contrast, a clear inhibition of MMP-1 production occurred after a treatment of FB with actinomycin D, apigenin, or PD 98059, respectively. Actinomycin D, which inhibits mRNA transcription, was used to distinguish between stabilized existing and newly transcribed mRNA (166). Apigenin is an inhibitor of mitogen-activated protein kinase (MAPK) signalling cascades (167), and PD98059 is a specific inhibitor of the ERK kinase MEK *in vitro* and *in vivo* (168). As shown in Fig. 10 A and B, actinomycin D completely inhibited up-regulation of MMP-1 mRNA confirming that newly transcribed mRNA is responsible for the increase of MMP-1 on the mRNA and protein level. Apigenin as well as PD98059 also inhibited the expression of MMP-1 indicating the necessity of the MEK-ERK signalling pathway for MMP-1 induction in FB.

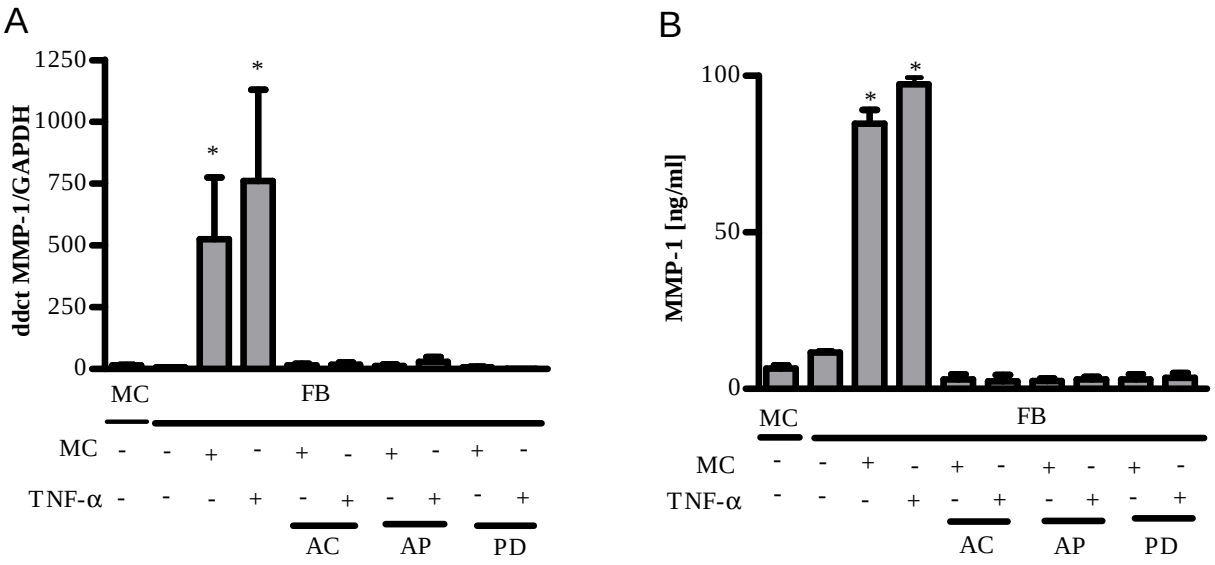


Figure 10*:* MMP-1 induction is blocked by the MEK inhibitor PD98059.

FB were treated with specific inhibitors actinomycin (AC) at 1 µmol/L, apigenin (AP) at 20 µmol/L, or PD 98059 (PD) at 1 µmol/L before treatment with MC or TNF (10 ng/ml). ***A.*** *MMP-1 mRNA expression was analyzed by real time RT-PCR.* ***B.*** *MMP-1 protein produced by FB was measured by ELISA (n=6, * $p<0.05$ compared to untreated FB, means ± SEM).*

3.5 MMP-1 induces loss of confluence in fibroblasts

MMP-1 has been considered to be a multifunctional modulator since it participates not only in turnover of collagen fibrils in the extracellular matrix but also in the cleavage of a number of non-matrix substrates and cell surface molecules, suggesting a role in cytokine activation and cellular trafficking (169).To test this hypothesis, confluent FB were treated with 100 ng/ml MMP-1. As shown in Fig. 11, MMP-1 protein induced a loss of confluence of FB after 24 h, and even more pronounced after 72h (Fig. 11 E) compared to controls (Fig. 11 D, E). Similar observations were made for FB after culture in direct cell-cell contact with MC (Fig. 11 C, F).

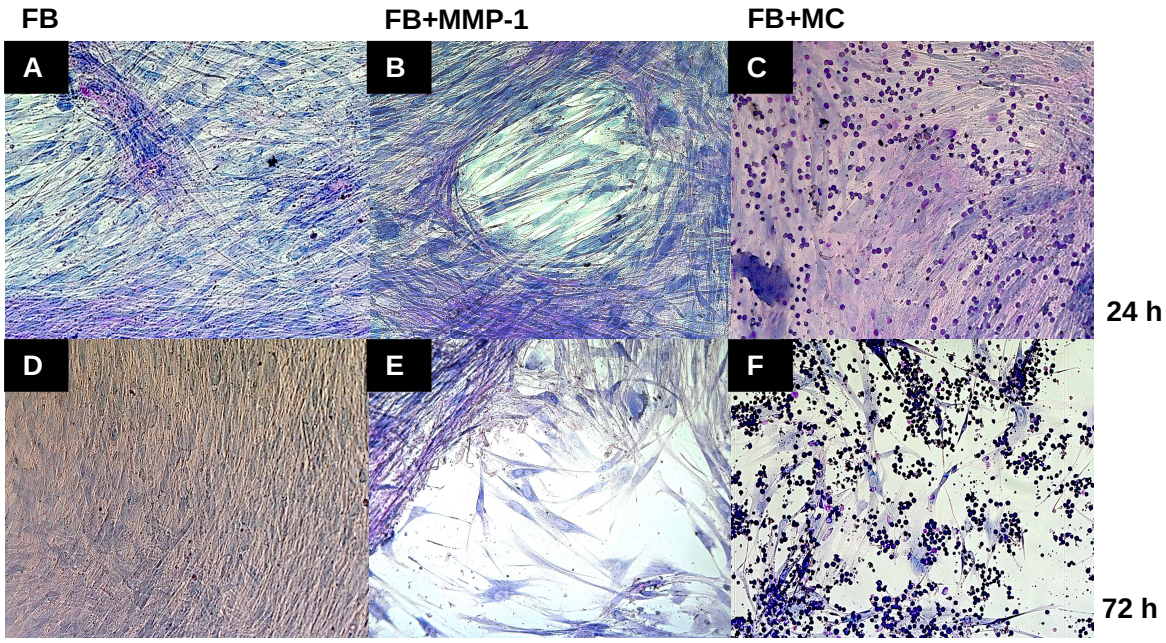


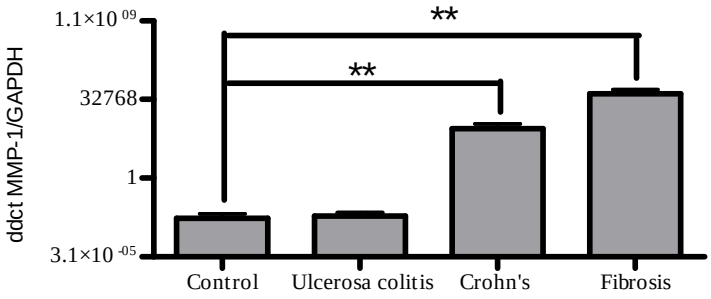
Figure 11*:* MMP-1 induces loss of confluence in FB.

*FB were incubated alone (**A, D**), with 100 ng/ml MMP-1 (**B, E**), or with 1x10^5 MC/ml (**C, F**) for 24 h (**A-C**) or 72 h (**D-E**).Cells stained by May Grünwald/Giemsa were shown.*

3.6 Analysis of biopsies derived from human gut

mRNA for MMP-1 was significantly increased in biopsies derived from patients suffering from fibrosis and CD, whereas mRNA for MMP-1 was unchanged in biopsies of patients with UC (Fig 12A). To confirm the mRNA data, immunohistochemical analyses were performed with an antibody against human MMP-1 (Fig 12 B).

A



B

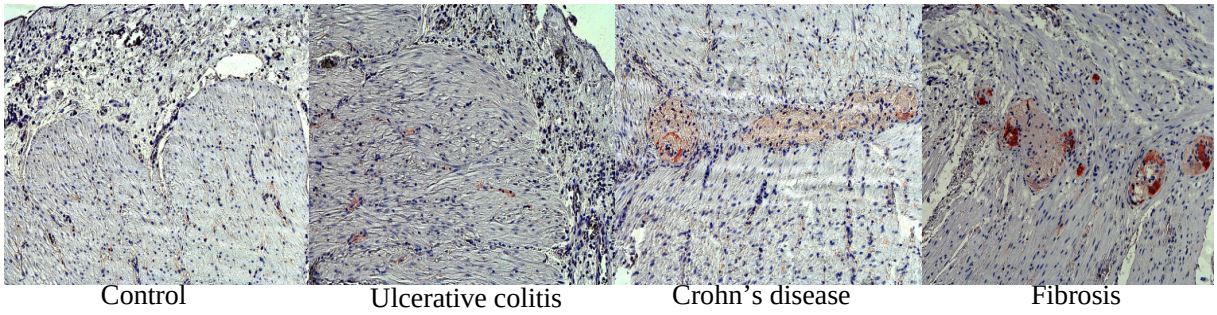


Figure 12: MMP-1 expression in the biopsies.

Biopsies derived from patients with ulcerative colitis, crohn´s Diseas and fibrosis were analyzed by means of immunohistochemistry ***A****, RNA isolated from biopsies were analyzed by real time-PCR (n=3, ** p <0,01 compared to control)* ***B****, Biopsies were prepared and labeled with an antibody against MMP-1.*

Discussion

4.1 IL-6 support human mast cell survival

In this work it is shown that IL-6 supports human MC survival independent on the previously known human MC growth factors SCF, IL-3, and IL-4. Human intestinal MC survive up to 3 weeks in co-culture with human intestinal FB, whereas MC mono-cultures die within a week (1). We could show that IL-6 is the factor responsible for MC survival produced by FB by demonstrating that 1) IL-6 supports MC survival for up to 3 weeks of culture as found for FB-dependent MC survival, and 2) MC survival could be reduced or even blocked using a neutralizing Ab against IL-6. In line with our results on intestinal MC, IL-6 has been described before to prolong the survival of cord blood derived MC in a dose-dependent manner (170). In contrast, the SCF-dependent development of CD34+ cord blood derived MC was inhibited by IL-6 (134). In a previous study of the group, it has been reported that MC survival in co-culture with FB was clearly enhanced in the presence of IL-1β or TNF (1, 78). Since neither IL-1β nor TNF affected MC survival directly, it was likely that both cytokines induce or enhance the production of MC survival factor(s) in FB. We could now provide an explanation for this phenomenon by showing that IL-1β or TNF induces IL-6 expression in FB, in line with studies of others on non-intestinal fibroblasts (171-173). Fitzgerald et al (174) reported recently that human lung FB express IL-6 in response to cellular membranes of the leukemic human mast cell line HMC-1 independent of the intercellular adhesion molecule -1 (ICAM-1), IL-1β, and TNF receptor. We found that human intestinal FB produce IL-6 in response to co-culture with human intestinal MC which are capable of producing a wide range of cytokines including IL-β and TNF (77). Interestingly, not only TNF and IL-1β but also specific MC mediators such as tryptase and histamine were capable of inducing IL-6 expression in intestinal FB.

The regulation of intestinal MC by intestinal FB differs from that of MC regulation by endothelial cells. We reported earlier that human umbilical vein endothelial cells (HUVEC) support MC growth almost exclusively by membrane-bound factors (SCF/c-kit and adhesion molecules VCAM-1/VLA-4) (153). In contrast, FB cause MC survival by releasing soluble IL-6, since transwell experiments in which MC were separated from FB yielded similar results with regard to MC recovery. Furthermore, FB supernatants also promoted MC growth, whereas FB sonicates had only little effect on MC survival (1). Compared to treatment of MC with SCF or co-culture of MC with HUVEC, intestinal FB were less effective in promoting MC survival (153, 175). This could be due to the lower potency of IL-6 to support MC survival compared to SCF. Noteworthy, the amounts of sSCF produced by intestinal FB were 10-100 times lower than those required for maintaining MC survival *in vitro* (1). Taken the *in vitro* findings together, MC mediators such as TNF, IL-1β, histamine and tryptase stimulate FB to produce IL-6 that, vice versa, supports MC survival; suggesting an close crosstalk between these two cell types in intestinal tissues.

4.2 Mast cells promote synthesis of MMP-1 by fibroblasts

MC are thought to play a role in the pathophysiology of fibrosis but the molecular basis for their intercellular interaction is basically unknown (146, 176, 177). Garbuzenko et al. (175) reported that sonicates of the leukemic MC line (HMC-1) increased human skin fibroblast proliferation, collagen synthesis, TIMP-2 and collagen gel contraction. The authors concluded, that MC have a direct effect on skin remodeling and fibrosis (175). Both leukemic (HMC-1) and dermal MC were found to express fibroblast growth factor 2, fibroblast growth factor 7, and heparin-binding epidermal growth factor at least at the mRNA level (178). A study in MC-deficient rats and mice aiming to evaluate the role of MC in the development of liver fibrosis shown that neither bile duct obstruction nor administration of carbon tetrachloride caused an increase in MC density at sites of fibrosis, which occured also in MC deficient mice. These data suggest that MC play no role in the development of liver fibrosis in rats and mice (179).

In contrast, I found that MC strongly up-regulated the formation of fibrolytic MMP-1 in FB whereas profibrogenic TIMP-1 and -2 as well as collagens were not affected by MC. MMP-1 is thought to be a key enzyme for interstitial fibrolysis, a process closely that is also related to tissue remodeling (178). This enzyme is also known to be a multifunctional modulator since it participates not only in the turnover of collagen fibrils in the extracellular space but also in the cleavage of a number of non-matrix substrates and cell surface molecules (163). We could show that cultured intestinal FB treated with MMP-1 lost their confluence. The same was true for FB co-cultured with MC supporting a role of MC-derived mediators in the up-regulation of MMP-1 in FB. Tasaki et al. (178), reported that human pancreatic periacinar myofibroblasts produce MMP-1 in response to the pro-inflammatory cytokines TNF and IL-1β. We could extend these observations for human intestinal FB by showing that not only TNF and IL-1β but also the specific MC mediators tryptase and histamine are

capable of inducing MMP-1 expression in intestinal FB. Moreover, we found that MC dependent MMP-1 expression in FB was mediated via the MEK/ERK branch of the MAPK pathway, but occurred independent of PKC or PI3K. These findings are in accordance with observations reported for human pancreatic periacinar myofibroblasts showing that inhibition of MEK/ERK, but not p38 MAPK and PKC, alters MMP-1 secretion (178) (Fig 13).

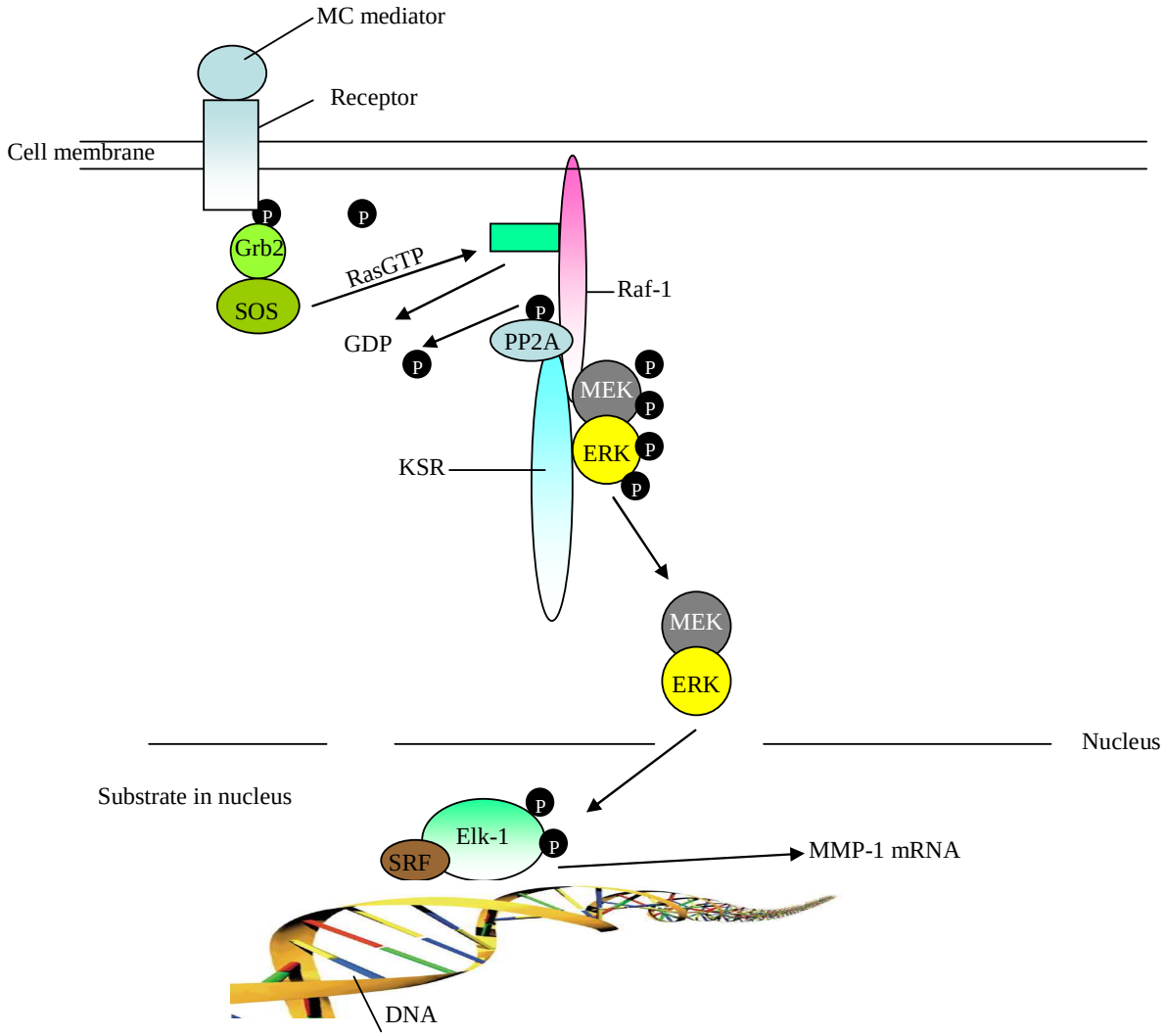


Figure 13: Organisation and function of the MEK-ERK pathway

Modified fromYeung *et al* (180)

Binding of MC mediators induces autophosphorylation (P) on tyrosine residues. These phosphotyrosines function as docking sites for signalling molecules including the Grb2-SOS complex, which activates the small G-protein Ras by stimulating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). This exchange elicits a conformational change in Ras, enabling it to bind to Raf-1 and recruit it from the cytosol to

the cell membrane, where Raf-1 activation takes place. Raf-1 activation is a multi-step process that involves the dephosphrylation of inhibitory sites by protein phosphatase 2A (PP2A) as well as the phosphorylation of activating sites by PAK (p21$^{rac/cdc42}$-activated kinase), Src-family and yet unknown kinase. Activated Raf-1 phosphorylates and activates MEK (MAPK/ERK kinase), which in turn phosphorylates and activates extracellular-signal-regulated kinase (ERK). The interaction between Raf-1 and MEK can be disrupted by RKIP (Raf kinase inhibitor protein; not shown). The whole three-tiered kinase cascade is scaffolded by KSR (kinase suppressor of Ras). Activated ERK has many substrates in the cytosol . ERK can also enter the nucleus to control gene expression by phosphorylating transcription factors such as Elk-1 and other Ets-family proteins.Grb2, growth-factor-receptor-bindingprotein 2; SOS, "Son of sevenless"; SRF, serum response factor

In vivo, we observed that mRNA expression of MMP-1 was significantly increased in fibrosis and CD but not in UC. This results together with our finding that MC induce MMP-1 expression in FB, these data correlate with the results of Gelbmann *et al* (90) that show an increase of MC in human biopsies from patients with CD and fibrosis but not in biopsies from patients with UC. These data could be helpful in the diagnosis of the disease and a better adaptation to the therapies.

4.3 Summary

The data of my doctoral thesis provide new insights into the mechanisms of interactions between MC and FB derived from human intestinal mucosa. MC mediators are capable of stimulating FB to produce IL-6 that vice versa promotes MC survival. Moreover, MC mediators are capable of inducing the expression of MMP-1 in FB, a key enzyme in fibrolysis. Taken together the data strongly suggest that MC may play an important role in fibrolysis and remodeling of damaged tissue rather than in fibrogenesis.

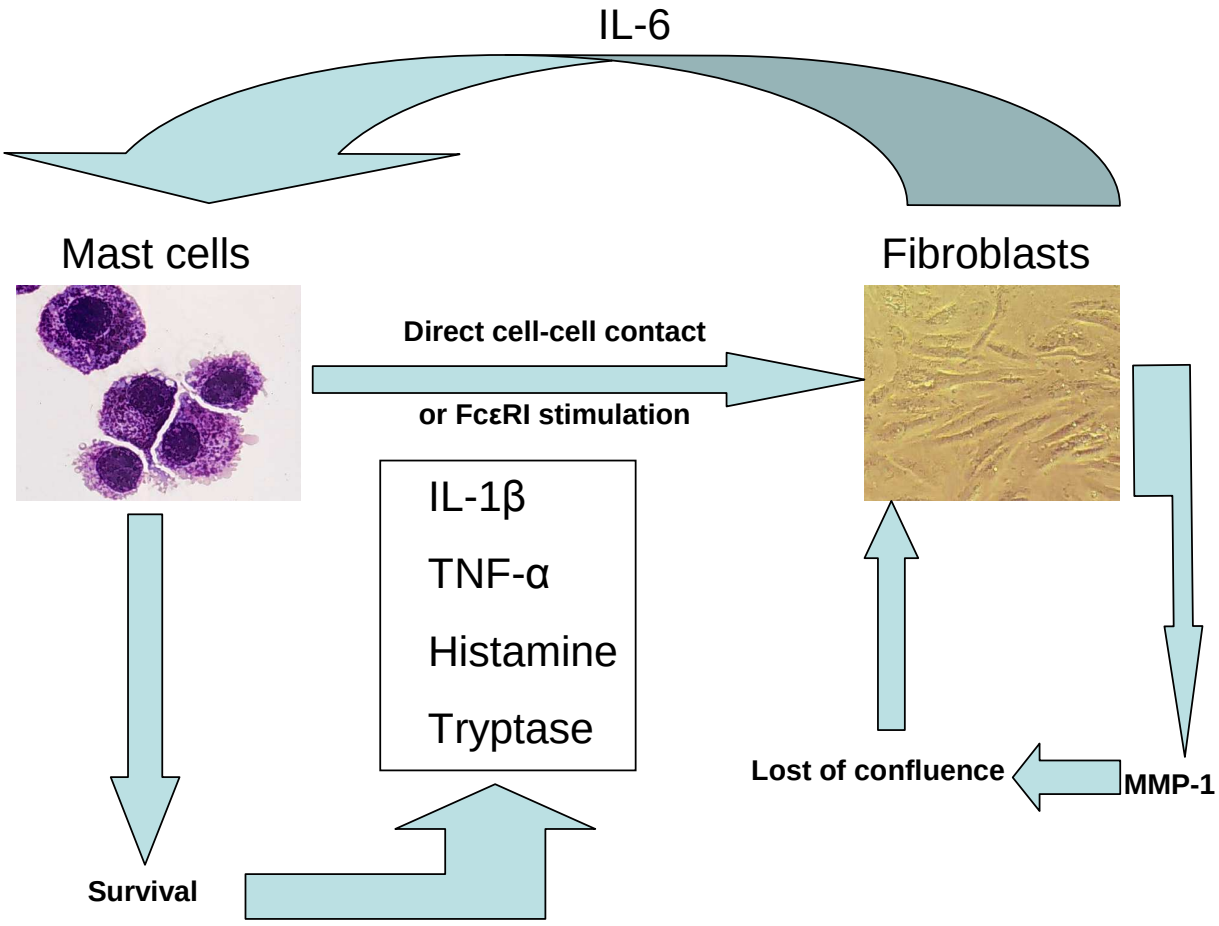


Figure 14: Summary of the work

MC mediators stimulated FB that produced IL-6 that induce the survival of the MC. Moreover stimulated FB produce MMP-1 that induce the lost of confluence of the FB.

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Curriculum vitae

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Publication list

8.1 Original publication

Montier Y, Lorentz A, Krämer S, Sellge G, Bauer M, Schuppan D, Bischoff SC "Central role of IL-6 and MMP-1 for cross talk between human intestinal mast cells and human intestinal fibroblasts**"** Journal of Immunology

8.2 Abstracts contributions

Montier Y, Lorentz A, Krämer S, Sellge G, Bauer M, Schuppan D, Bischoff SC „Cross talk between human intestinal mast cells and human intestinal fibroblasts" Trieste 2007

Montier Y, Lorentz A, Krämer S, Sellge G, Bauer M, Schuppan D, Bischoff SC **"**Intestinal mast cell mediators strongly upregulate expression of MMP-1 in intestinal fibroblasts**"** ImmunoRio 2007

Montier Y, Lorentz A, Sellge G, Schuppan D, Bischoff SC „Mast cells and intestinal fibroblasts" Helsinki 2006

Montier Y, Lorentz A, Sellge G, Schuppan D, Bischoff SC „Human intestinal mast cells induces expression of metalloproteinase 1 in human intestinal fibroblasts" EAACI 2006

Montier Y, Lorentz A, Sellge G, Schuppan D, Bischoff SC „Human intestinal mast cells induces expression of metalloproteinase 1 in human intestinal fibroblasts" Paris 2006

Montier Y, Lorentz A, Sellge G, Schuppan D, Bischoff SC „ Mast cells and intestinal allergy, inflammatory bowel disease and intestinal fibrosis" Stockholm 2005

Erklärung/Declaration

Ich erkläre, dass ich die der Universität Hohenheim zur Promotion eingereichte Dissertation mit dem Titel

„Wechselwirkungen zwischen humanen Darmmastzellen und humanen Darmfibroblasten“

selbständig verfasst und die benutzten Hilfsmittel un Quellen angegeben habe.

I declare submit to the university of Hohenheim for obtain the title of PhD the manuscript with the title

“Interactions between human intestinal mast cells and human intestinal fibroblasts”

Stuttgart 24.10.2007