The guanylate binding protein-1: a molecular marker of the inflammatory cytokine-activated phenotype of endothelial cells

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Clara Lubeseder-Martellato
aus Venedig, Italien

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ABBREVIATIONS

MAb	monoclonal antibody
AGF	angiogenic growth factors
Amp	ampicillin
Ang-2	angipoietin-2
APAAP	alkaline phosphatase anti- alkaline phosphatase
AP	alcaline phosphatase
ATP	adenosine 5'-trisphosphate
bp	base pair(s)
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
ca.	circa
CLSM	confocal laser scanning microscopy
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamino-2-phenylindole
DMEM	Dulbeccos minimum essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	1,4-dithiothreitol
E. col.	*Escherichia coli*
EDTA	thylenediaminetetraacetic acid
EEM	erythema exudativum multiforme
g	ground acceleration;
GAPDH	glyceraldehyd phosphate dehydrogenase
GBP-1	human guanylate binding protein 1
gr	gram(s)
eGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
FPLC	fast performance liquid chromatography
h	hour(s)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
HUVEC	primary human umbilical-vein endothelial cells
IC	inflammatory cytokines
IFN-α	interferon-α,
IFN-γ	interferon-gamma
IgG	immunoglobulin G
IL	interleukin
IPTG	isopropyl-ß-thiogalactopyranoside
IP-10	interferon.inducible protein-10 kilodaltons
kb	kilobase(s)
kDa	kilodalton
KS	Kaposi´s sarcoma
l	litre(s)
LB	Luria broth
LDH	lactate dehydrogenase
LTR	long terminal repeat
MW	molecular weight
µ	micro

M	molar
MAb	monoclonal antibody
MCP-1	monocyte chemoattractant protein-1
mGBP	mouse guanylate binding protein 1
min	minute(s)
MIP-1β	macrophage inflammatory protein-1 beta
ml	millilitres
dMVEC	dermal human macro-vascular umbilical vein endothelial cells
µm	micrometer
mM	millimolar
NAD	nicotin adenin dinucleotide
NiNTA	nichel nitrilotriacetic
nm	nanometer
NP40	nonylphenyl polyethyleneglycol
NSF	N-ethylmaleimide-sensitive fusion protein
OD	optical density
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF B/B	platelet-derived growth factor
PF4	platelet factor 4
PNPP	p-nitrophenil-pirophosphate
psi	pounds per square inch, 1 psi = 6897 Pa
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
s	second(s)
SD	standard deviation
SDF-1α	stromal cell-derived factor-1 alpha
SDS	sodium dodecyl sulfate
sol.	solution
ssDNA	single stranded DNA
TAE	tris-acetate-EDTA
TCA	trichloroacetic acid
TCEP	tris(2-carboxyethyl) phosphine
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TNF-α	tumor necrosis factor alpha
Tris	tris-(hydroxymethyl)-aminomethane
Triton-X100	octylphenolpoly (ethyleneglycolether)$_n$
TRS9	target retrieval solution pH 9
Tween 20	polyoxyethylene-sorbitanmonolaurate
U	unit(s)
UV	ultra violet
V	volt
VEGF	vascular endothelial growth factor
v/v	volume per volume
vol.	volume(s)
w/v	weight per volume

SUMMARY

The endothelium is among the largest organs in the body. Stimuli originating from the blood or from neighbouring cells, like inflammatory cytokines (IC), lead to structural and functional alterations of vascular endothelial cells (EC). These alterations are often referred to as "EC activation". Activated EC play a key role in different physiological processes like during immune response, in menstruation and in pathological processes like inflammation, allergy, viral infections, atherosclerosis and tumour angiogenesis.

The human guanylate binding protein-1 (GBP-1) is a protein of the family of large GTPases. GBP-1 is characterized by a high turnover GTPase activity. Previous work showed that GBP-1 mRNA expression is induced by IC in EC and that GBP-1 is the specific mediator of the anti-proliferative effect of IC on EC *in vitro*.

The main goals of this work were first, to investigate whether GBP-1 may be a molecular marker of IC-activated EC at the protein level *in vitro*. Second, to investigate GBP-1 expression in human healthy and/or disease tissues and to determine whether GBP-1 may be a molecular marker of IC-activated EC *in vivo*.

To this goal mono- and poly-clonal antibodies against GBP-1 were generated. *In vitro* studies showed that GBP-1 expression in EC is induced by IFN-γ, IFN-α, IL-1α, IL-1β or TNF-α but not by other cytokines, chemokines or growth factors. Moreover, simultaneous addition of bFGF and VEGF and IC reduced the IC-induced GBP-1 expression. This indicated that GBP-1 characterizes cells that are preferentially exposed to IC.

In vivo studies using immunohistochemistry and immunofluorescence showed that GBP-1 expression is highly associated with vascular EC in a broad range of human tissues. This was confirmed by the simultaneous detection of GBP-1 and the EC-associated marker CD31. Notably, GBP-1 expression was undetectable in healthy skin. In contrast, GBP-1 was highly expressed in vessels of skin diseases with a high inflammatory component including psoriasis, adverse drug reactions and Kaposi's sarcoma. This indicated that GBP-1 characterizes IC-activated EC *in vivo*. Further immunohistochemical studies on Kaposi's sarcoma demonstrated that GBP-1 expression and EC cell proliferation are inversely related. This indicated that GBP-1 may also mediate the anti-proliferative effect of IC on EC *in vivo.*

Finally, GBP-1 was found to be secreted by EC stimulated with IFN-γ and IFN-α *in vitro*. This finding was confirmed by immunoprecipitation of GBP-1 from cell culture supernatants and by a novel ELISA developed for the detection of GBP-1 in solution. Further characterization of the mechanism of secretion demonstrated that GBP-1 release is due to an

energy-dependent mechanism and is not due to cell death. Most importantly, circulating GBP-1 could be detected in increased concentrations in the blood of patients that were subjected to IFN–α-therapy or in patients with inflammatory diseases.

These findings indicated that GBP-1 is a novel marker of inflammatory vessel activation. Specifically, the serological detection of GBP-1 may open new perspectives for the early detection of inflammatory activation of EC in patients with inflammatory diseases.

INTRODUCTION

1 Function of the quiescent endothelium

The endothelium is among the largest organs in the body. In an adult it covers a surface of almost 1000 m^2 with a mass of 720 g (Cines, *et al.* 1998; Bachetti, *et al.* 2000). The endothelium is located at the interface between the blood and the vessel wall and consists of quiescent endothelial cells (EC).

The endothelium in healthy persons consists of EC that are in close contact and form a non-adhesive layer that prevents blood cell interaction with the vessel wall as blood moves through the vessel lumen (Augustin, *et al.* 1994; Cines, *et al.* 1998). In this framework, EC are the only cells known to be actively antithrombotic (Tan, *et al.* 1999). In addition, the endothelium serves both as a barrier and as a regulator of transvascular diffusion of liquids and solutes (Augustin, *et al.* 1994; Cines, *et al.* 1998; Datta, *et al.* 2001).

The pathway of liquids and solutes across EC utilises vesicle trafficking (for example caveolae), which is regulated by endocytotic and exocytotic events (Niles, *et al.* 1999). It has been shown that the phosphoinositide metabolism regulates endocytosis and that exocytosis is regulated by the soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptors machinery (SNAREs) (Niles, *et al.* 1999). Most SNAREs are C-terminally anchored integral membrane proteins capable of entering into an interaction with other SNARE proteins. SNARE proteins are believed to mediate most, if not all, cellular membrane fusion events (Tooze, *et al.* 2001; Schekman 2002). However, the exact mechanisms of endocytosis and exocytosis in EC are still unclear.

In addition, the endothelium has secretory functions (Bachetti, *et al.* 2000). The trafficking of secretory proteins within eukaryotic cells is achieved by the capture of cargo and targeting molecules into vesicles. Distinct coat proteins mediate each budding event. These coating proteins shape the transport vesicles and select the desired set of cargo molecules (Schekman 1998; Schekman 2002). Transport vesicles are characterized by such coating proteins and by sphingolipid-cholesterol rafts, that are insoluble in the detergent Triton-X100 at 4 °C (Simons, *et al.* 1997).

Regulated secretion of transmigrated vesicles and the release of EC encoded factors provide a mean by which EC can rapidly and selectively alter the microenviroment of individual vascular beds and modulate different correlated processes as listed below (Datta, *et al.* 2001):

(1) Coagulation is regulated via secretion of P-selectin, von Willebrand factor, tissue plasminogen activator, plasminogen activator inhibitor, nitric oxide and multimerin (Hayward*, et al.* 1998; Hayward*, et al.* 1999; Datta*, et al.* 2001; Nilius*, et al.* 2001).

(2) Vaso-dilatation and -constriction are regulated via secretion of prostacycline, endothelin-1, angiotensin II, superoxide radicals (Harrison*, et al.* 1995; Bechard*, et al.* 2000; Datta*, et al.* 2001; Nilius*, et al.* 2001; Wang*, et al.* 2002).

(3) Inflammation is regulated via secretion of IL-8, TNF-α and superoxide radicals (Kaplanski*, et al.* 1997; Datta*, et al.* 2001; Nilius*, et al.* 2001).

(4) Cell proliferation is regulated via secretion of aFGF and bFGF (Swinscoe*, et al.* 1992; Friesel*, et al.* 1999; Tarantini*, et al.* 2001; Prudovsky*, et al.* 2002).

This indicates that the endothelium is involved in many different processes in the human body.

Of note, EC have often specialized functions, depending on the tissue in which they are located. Therefore, vascular endothelial cells reveal structural and functional heterogeneity (Kuzu*, et al.* 1992; Girard*, et al.* 1999; Bachetti*, et al.* 2000). For example, heterogeneity has been shown in the activation of micro- and macro-vascular EC in response to growth factors with respect to activation of protein kinase C and expression of adhesion molecules (Kuzu*, et al.* 1992; Swerlick*, et al.* 1992; Mason*, et al.* 1997).

2 Pathophysiological activation of the endothelium during inflammation

However, the endothelium can react in a dynamic manner and, under appropriate stimulation, EC can undergo profound changes leading to structural and functional alterations. These alterations are often referred to as "endothelial cell activation" (Pober*, et al.* 1986; Cotran*, et al.* 1988; Pober 1988; Cotran*, et al.* 1990; Augustin, *et al*. 1994). Endothelial cell activation plays a key role for example during inflammation.

Inflammation is an important component in many diseases including atherosclerosis, tumor metastasis, infection, trauma, chemical and metabolic injury (Cotran*, et al.* 1990; Siegel*, et al.* 1997; Boehm*, et al.* 1998; Livni*, et al.* 1999; Tan*, et al.* 1999; Baumgartl*, et al.* 2001; Roesen*, et al.* 2001; Cascieri 2002).

In the course of inflammation the activation of the endothelium involves a local activation response of EC to injury and/or infection (Swerlick*, et al.* 1993; Siegel*, et al.* 1997; Biedermann 2001). The inflammatory process can be divided in: (**i**) an acute vascular

response within the first seconds of tissue injury characterized by vasodilatation, increased capillary permeability and alterations of the vascular endothelium. (**ii**) An acute cellular response within the first hours, characterized by the appearance of granulocytes in the tissue. (**iii**) In the case of severe damage a chronic cellular response of EC is observed within the next days, regulating the recruitment of a mononuclear cell infiltrate composed of macrophages and lymphocytes to the site of injury. In the case of wound healing sprouting of vessels occurs in the damaged area (Tan*, et al.* 1999; Detmar*, et al.* 1998).

Activation of the endothelium is caused by the injurious stimulus itself, or by inflammatory cytokines generated in response to the stimulus (Cotran*, et al.* 1988; Pober 1988; Cotran*, et al.* 1990; Augustin*, et al.* 1994; Tan*, et al.* 1999). The goal of inflammatory EC activation is to limit the damage induced by the external stimuli **(i)** *via* the recruitment of blood cells (leukocytes) to the site of injury, **(ii)** by initiating blood coagulation and **(iii)** by healing and promoting repair for recovery of function (Shimizu*, et al.* 1992; Swerlick*, et al.* 1992; Luscinskas*, et al.* 1994; Haraldsen*, et al.* 1996; Robson*, et al.* 1997; Jung*, et al.* 1998; Tan*, et al.* 1999).

2.1 Vessel sprounting (angiogenesis)

Angiogenesis is defined as the process of generating new capillary blood vessels from pre-existing ones (Folkman 1995; Cines*, et al.* 1998). Angiogenesis can occur in association with inflammation for example during wound healing (Breier*, et al.* 1992; Folkman 1995; Detmar*, et al.* 1998).

Key features of the process of angiogenesis are first, EC proliferation induced by angiogenic growth factors and second, EC invasion of the extracellular matrix (ECM) (Montesano*, et al.* 1986; Schweigerer*, et al.* 1987b; Sato*, et al.* 1988; Leung*, et al.* 1989; Pepper*, et al.* 1992; Melder*, et al.* 1996). The effect of angiogenic growth factors is described in detail below.

Invasion of ECM involves both motility of EC and proteolysis of the ECM by proteases secreted by EC (Fisher*, et al.* 1994; Moses 1997; Hiraoka*, et al.* 1998). Angiogenic proteolysis is dependent on matrix metalloproteinases that degrade the tissue in front of the sprouting vessels and allow EC to migrate towards the diseased tissue (Pepper*, et al.* 1992; Pepper 2001; Silletti*, et al.* 2001; Vihinen*, et al.* 2002). Invasion of EC is accompanied by an increased expression of adhesion molecules that in turn increases adhesion of blood cells to EC (Klein*, et al.* 1993; Melder*, et al.* 1996; Detmar*, et al.* 1998; Lu*, et al.* 1999; Kim*, et al.* 2001).

During the last step in angiogenesis, EC form blood vessels through a process called "capillary formation". Finally, individual blood vessels are connected and a vascular system is formed.
Angiogenic growth factors (AGF) such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are the most potent modulators of angiogenesis.

2.2 The role of angiogenic growth factors in endothelial cell activation

2.2.1 Basic fibroblast growth factor (bFGF)

bFGF is a non-glycosylated heparin-binding factor. bFGF is produced mainly by EC, in particular it is released after tissue injuries and during inflammation (Westermann, *et al.* 1990). *In vivo*, bFGF expression has been detected in different human tumors (Chodak, *et al.* 1988; Tanimoto, *et al.* 1991; Nakamoto, *et al.* 1992; Dirix, *et al.* 1997; Chopra, *et al.* 1998).
bFGF can induce proliferation, chemotaxis and migration of EC *in vitro* (Montesano, *et al.* 1986; Sato, *et al.* 1988; Gospodarowicz 1991). However, bFGF does not only activate proliferation of EC, but also of many other cells like fibroblasts, myoblasts, osteoblasts, neuronal cells, keratinocytes and chondrocytes (Westermann, *et al.* 1990; Gospodarowicz 1991). In addition it up-regulates the expression of integrins and proteinases in EC (Moscatelli, *et al.* 1986; Gospodarowicz 1991; Moses 1997).
In vivo, bFGF has been suggested to play a major role in tumor angiogenesis (Gospodarowicz 1984; Thomas, *et al.* 1985; Gospodarowicz 1991).

2.2.2 Vascular endothelial growth factor (VEGF)

VEGF is also an heparin-binding factor. VEGF consists of a family of different factors (VEGF-A, -B, -C, -D and PIGF) that are generated by differential splicing (Maglione, *et al.* 1991; Grimmond, *et al.* 1996; Joukov, *et al.* 1996; Neufeld, *et al.* 1996; Olofsson, *et al.* 1996; Poltorak, *et al.* 1997; Yamada, *et al.* 1997; Neufeld, *et al.* 1999; Meyer, *et al.* 1999).
VEGF is produced by macrophages, lung epithelial cells, kidney epithelial cells, follicular cells in the pituitary, corpus luteum cells, aortic smooth muscle cells and tumor cells (Leung, *et al.* 1989; Brown, *et al.* 1992). In addition, *in vivo* VEGF is expressed in many different human tumors (Dirix, *et al.* 1997; Fujisaki, *et al.* 1998; Landriscina, *et al.* 1998; Salven, *et al.* 1998; Samaniego, *et al.* 1998).

VEGF induces proliferation of EC, but in contrast to bFGF it almost selectively activates micro- and macro-vascular EC (Folkman, *et al.* 1987; Keck, *et al.* 1989; Leung, *et al.* 1989; Wilting, *et al.* 1993). Moreover, VEGF increases blood vessel permeability and induces chemotaxis and migration of EC and monocytes (Nicosia, *et al.* 1994; Barleon, *et al.* 1996; Esser, *et al.* 1998; Thurston, *et al.* 1999; Kim, *et al.* 2001).

VEGF has been shown to regulate angiogenesis in different *in vivo* models (Phillips, *et al.* 1990; Breier, *et al.* 1992; Kim, *et al.* 1993; Wilting, *et al.* 1993; Phillips, *et al.* 1994; Thurston, *et al.* 1999).

Notably, bFGF and VEGF interact synergistically in the induction of EC proliferation *in vitro* and in the induction of angiogenesis *in vivo* (Pepper, *et al.* 1992; Cornali, *et al.* 1996; Melder, *et al.* 1996).

2.3 Recruitment of leukocytes

Activated EC play a threefold role in the recruitment of leukocytes to the site of inflammation (Swerlick, *et al.* 1993; Ley 1996). First, activated EC express adhesion molecules that allow adhesion of leukocytes at sites of injury like the selectin family of adhesion molecules, intercellular adhesion molecules (ICAMs), vascular adhesion molecule-1 (VCAM-1), integrines and platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31) (Pober, *et al.* 1986; Bevilacqua, *et al.* 1989; Cavender, *et al.* 1991; Shimizu, *et al.* 1992; Luscinskas, *et al.* 1994; Jung, *et al.* 1998; Tan, *et al.* 1999). Second, activated EC attract leukocytes via the expression of chemokines (Siegel, *et al.* 1997; Krishnaswamy, *et al.* 1999). Third, activated EC release cytokines (e.g. IL-1, IL-6, IL-8) in response to leukocyte derived molecules. These cytokines are activating cells in the surrounding and *via* this amplify the response of EC (Bevilacqua, *et al.* 1989; Cavender, *et al.* 1991; Biedermann 2001).

The cytokines that appear to have the most profound effect on EC during inflammation are the inflammatory cytokines (IC): interferon-γ (IFN-γ), interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (Pober 1988; Tan, *et al.* 1999). IC induce the expression of adhesion molecules both on EC and lymphocytes and are potent inhibitors of EC proliferation (Frater-Schroder, *et al.* 1987; Friesel, *et al.* 1987; Schweigerer, *et al.* 1987a; Cozzolino, *et al.* 1990; Swerlick, *et al.* 1992; Haraldsen, *et al.* 1996).

2.4 The role of inflammatory cytokines in endothelial cell activation

2.4.1 Interferon-gamma (IFN-γ)

IFN-γ is a dimeric, glycosylated protein and can exist in a form associated with the extracellular matrix [reviewed in (Farrar, *et al.* 1993; Goodbourn, *et al.* 2000)]. IFN-γ is produced mainly by T-cells and natural killer cells, but also by B-cells. The synthesis of IFN-γ can be induced in these cells by antigens and mitogens like IL-2, bFGF and EGF (Fiorelli, *et al.* 1998; Sirianni, *et al.* 1998; Cooper, *et al.* 2001).

A number of receptors have been described for IFN-γ (Rubinstein, *et al.* 1987; Schreiber, *et al.* 1992; Farrar, *et al.* 1993; Pestka, *et al.* 1997; Lambert, *et al.* 2000). They are expressed on all types of human cells with the exception of mature erythrocytes (van Loon, *et al.* 1991). IFN-γ/receptor complexes are rapidly internalised by endocytosis (Sadir, *et al.* 2000).

IFN-γ has antiviral and antiparasitic activities. IFN-γ inhibits the proliferation of a number of transformed and normal cells. In particular IFN-γ inhibits EC proliferation (Friesel, *et al.* 1987; Holzinger, *et al.* 1993; Jaramillo, *et al.* 1995; Neary, *et al.* 1996; Anderson, *et al.* 1999; Goodbourn, *et al.* 2000). In addition, IFN-γ increases the adhesion of T-cells by increasing the expression of ICAM-1 both on micro- and macro-vascular EC (Ruszczak, *et al.* 1990; Thornhill, *et al.* 1990). The most abundant IFN-γ-induced proteins are two families of GTPases of 65-kDa and 47-kDa [reviewed in (Boehm, *et al.* 1998)].

2.4.2 Interleukin-1 (IL-1)

IL-1 appears in two functionally almost equivalent forms that are encoded by two different genes with identical molecular weight (17 kDa): IL-1α and IL-1β (March, *et al.* 1985). IL-1α and IL-1β are both synthesized as precursors proteins of approximately 35 kDa. The mature proteins are released by the precursor protein by proteolytic cleavage (Black, *et al.* 1989; Beuscher, *et al.* 1990). The IL-1α precursor protein, but not the IL-1β, is biologically active (Mosley, *et al.* 1987; Beuscher, *et al.* 1990). The intracellular precursors of IL-1α and IL-1β do not contain a recognisable hydrophobic secretory signal sequence that may trigger secretion of the protein by classical secretory pathways involving the endoplasmic reticulum/Golgi apparatus (Rubartelli, *et al.* 1990; Tarantini, *et al.* 2001).

IL-1 is predominantly secreted by monocytes, but also by activated macrophages from different sources (alveolar macrophages, Kupffer cells, adherent spleen and peritoneal macrophages) and by many other cells (peripheral neutrophil granulocytes, EC, fibroblasts, smooth muscle cells, keratinocytes, Langerhans cells of the skin, osteoclasts, astrocytes, epithelial cells of the thymus and the cornea, T-cells, B-cells and NK-cells) (Hober*, et al.* 1989; Beuscher*, et al.* 1990).

Both forms of IL-1 bind to the same receptor. IL-1α and IL-1β show also very similar biological activities and are biologically more or less equivalent (Dower*, et al.* 1990; Symons*, et al.* 1991; Dinarello 2000).

IL-1 promotes the proliferation and the synthesis of immunoglobulins of B-cells, supports the monocyte-mediated tumor cytotoxicity and induces tumor regression (Norioka*, et al.* 1994). In addition; IL-1 causes many alterations of endothelial functions: it increases the adhesion of leukocytes, monocytes, neutrophils and B-cells by enhancing the expression of adhesion molecules such as ICAM-1, VCAM-1 and endothelial-leukocyte adhesion molecule (ELAM) (Swerlick*, et al.* 1992; Haraldsen*, et al.* 1996). IL-1 is also a strong chemo-attractant for leukocytes (Pober*, et al.* 1986; Larrick*, et al.* 1988; Last-Barney*, et al.* 1988; Pober 1988; Cotran*, et al.* 1990; Cavender*, et al.* 1991; Swerlick*, et al.* 1992; Haraldsen*, et al.* 1996; Dinarello 1996; Biedermann 2001). In addition, IL-1 decreases the expression of von Willebrand factor (vWf) and it increases the capability of EC to form tubule-like structures (Romero*, et al.* 1997). In particular IL-1 inhibits EC proliferation *in vivo* and *in vitro* (Cozzolino*, et al.* 1990; Holzinger*, et al.* 1993). In addition, IL-1 promotes thrombotic processes and attenuates anti-coagulatory mechanisms for example, by down-regulation of the expression of membrane-associated thrombomodulin (Maruyama*, et al.* 1989; Tan*, et al.* 1999)

Of note, IL-1 can interact synergistically with IFN-γ and TNF-α in the regulation of inflammatory reactions (Pober*, et al.* 1986; Last-Barney*, et al.* 1988; Holzinger*, et al.* 1993).

2.4.3 Tumor necrosis factor-alpha (TNF-α)

Human TNF-α is a non-glycosylated protein of 17 kDa that is maturated from a 233 amino acids precursor protein (Pennica*, et al.* 1984). TNF-α is secreted by macrophages, monocytes, neutrophils, CD4+ T-cells, NK-cells, different transformed cell lines, astrocytes, microglial, smooth muscle cells, and fibroblasts after exposure to bacterial lipopolysaccharides (Hober*, et al.* 1989; Vyakarnam*, et al.* 1991).

Receptors for TNF-α are expressed on all somatic cell types with the exception of erythrocytes. In addition, truncated soluble forms of the receptor have been found (Nophar, *et al.* 1990). TNF-receptor densities on the cell surface are decreased by IL-1 and tumor promoters such as phorbol esters. In contrast IFN-α, IFN-β, and IFN-γ increase IFN-γ-receptor density on the cell surface (Nedwin, *et al.* 1985; Pandita, *et al.* 1992).

TNF-α, similarly to IL-1, shows a wide spectrum of biological activities [reviewed in (Larrick, *et al.* 1988; Tracey, *et al.* 1993)]. In combination with IL-1, TNF-α induces many different effects on EC. It inhibits anticoagulatory mechanisms and promotes thrombotic processes by decreasing the expression of membrane thrombomodulin (Last-Barney, *et al.* 1988; Bevilacqua, *et al.* 1989; Biedermann 2001). TNF-α increases the adhesion of leukocytes to the endothelium (Larrick, *et al.* 1988; Pober 1988; Cotran, *et al.* 1990). TNF-α is also a potent chemoattractant for neutrophils and increases their adherence to the endothelium (Haraldsen, *et al.* 1996). In addition, TNF-α inhibits the growth of EC *in vitro* (Frater-Schroder, *et al.* 1987; Schweigerer, *et al.* 1987a). In contrast, TNF-α is a potent promoter of angiogenesis *in vivo* (Frater-Schroder, *et al.* 1987; Montrucchio, *et al.* 1994). This may be mediated *via* the recruitment of monocytes that in turn secret VEGF and bFGF. TNF-α can interact synergistically with IL-1β in the regulation of inflammatory reactions (Pober, *et al.* 1986; Last-Barney, *et al.* 1988).

Altogether, IC are important mediators of inflammatory ructions that have a profound effect on the activation of EC.

2.5 The role of inflammatory cytokines in inflammatory skin diseases

Inflammatory processes are clinically most apparent and most easily accessible when they occur in the skin. The skin is highly vascularized and each dermal papillae is addressed by a single capillary loop with arterial and venous vessels (Figure 1). Therefore in inflammatory diseases of the skin commonly an inflammatory activation of the underlying blood vessels is observed.

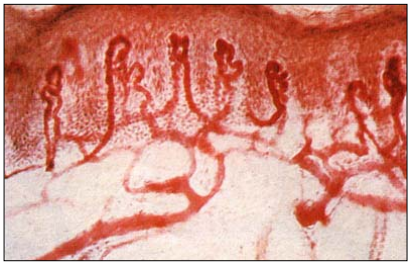


Figure 1: Healthy skin. *Cross section of the skin surface. In each dermal papillae a loop of capillary vessels is visible. From http://sprojects.mmi.mcgill.ca/dermatology/vessels.htm*

In this work skin diseases with a prominent inflammatory component were analyzed. The spectrum of different diseases studied included adverse drug reactions of the skin, psoriasis, urticaria, atopic dermatitis, erythema exudativum and Kaposi´s sarcoma. All these diseases are characterized by a local inflammatory response involving infiltration of inflammatory cells into the tissue and/or a local or systemic increase of IC concentrations.

2.5.1 Adverse drug reactions of the skin

Adverse drug reactions of the skin are mostly due to an allergic reaction to an applied drug. An allergic reaction causes an increase of IgE antibodies. IgE antibodies cause release of contents of mast cells that in turn activate EC and increase vascular permeability. Histologically drug reactions are characterized by vacuolar alteration of the basal layer, fibrosis of the papillary dermis and infiltrated lymphocytes (Figure 2). In particular, in individuals affected by an adverse drug reaction a strong IL-12 and IFN-γ reactivity of infiltrated T-cells has been reported (Yawalkar*, et al.* 2000).

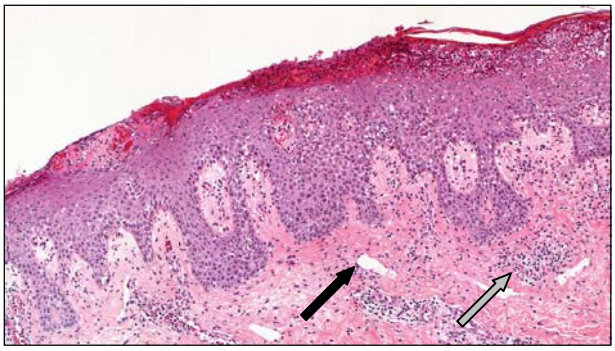


Figure 2: Histology of adverse drug reaction of the skin*. Staining of a skin tissue section by hematoxilin/eosin. Blood vessels (black arrow) and infiltrated lymphocytes (gray arrow) are indicated. Magnification: x 100. With permission from: Atlas of dermatology, Feith et al.,*

2.5.2 Psoriasis

Psoriasis is an inflammatory skin disorder which is characterized by a marked hyperproliferation of keratinocytes in association with an increased vascularization of the skin, fibroblasts activation and T-cell mediated inflammation. Trigger factors can be stress, infections or drugs. Histologically psoriasis is characterized by a thickened epidermis and highly vascularized dermal papillae (Figure 3).

It has been shown that in psoriatic lesions infiltrated leukocytes (mainly activated T-cells) produce IFN-α, IFN-γ and TNF-α (Gomi*, et al.* 1991; Kapp 1993; Chodorowska 1998).

Moreover, in psoriatic skin also a significant increase of IFN-γ producing mast cells has been found (Ackermann, *et al.* 1999).

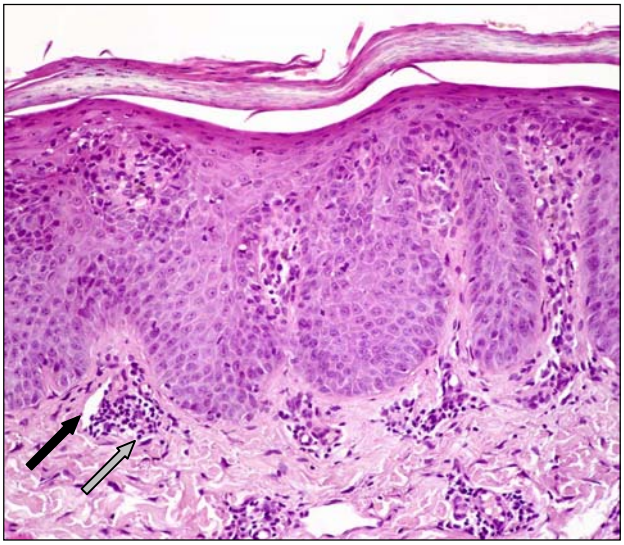


Figure 3: Histology of psoriasis*. Staining of a psoriasis tissue section by hematoxilin/eosin. Blood vessels (black arrow) and infiltrated lymphocytes (gray arrow) are indicated. Magnification: x 200. With permission from: Atlas of dermatology, Feith et al.*

2.5.3 Urticaria

Urticaria is caused by the release of histamine from mast cells that causes leakage of vessels, leading to angioedema. Chronic forms of urticaria are sometimes associated with vasculitis (an inflammation of the blood vessels that can occur in many other diseases). The aetiology can be a hypersensitive reaction to various stimuli: allergic (IgE, food or drugs) or non-allergic (physical stimuli like cold, or pressure). Histologcally urticaria is characterized by angioedema and by a perivascular infiltrate containing neutrophils and/or eosinphiles and an increased amount of intradermal mastocytes (Figure 4). It has been shown that in patients with drug-induced urticaria the occurrence of positive IFN-γ responses of peripheral blood lymphocytes to the suspected drug was significantly higher than in controls (Livni, *et al.* 1999).

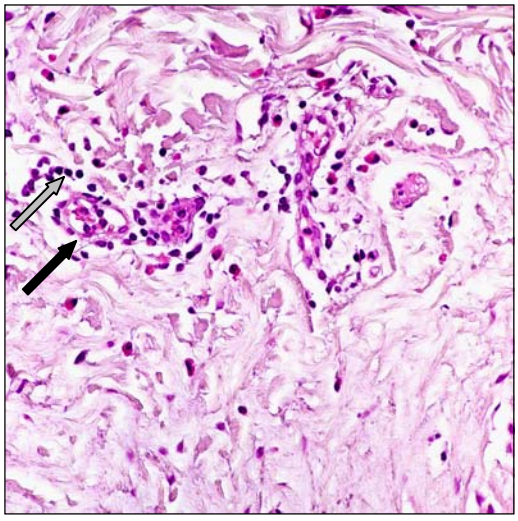


Figure 4: Histology of urticaria*. Staining of an urticaria tissue section by hematoxilin/eosin. Blood vessels (black arrow) and perivascular inflammatory cell infiltrate (gray arrow) are indicated. Magnification: x 400. With permission from: Atlas of* dermatology, Feith et al.

2.5.4 Atopic dermatitis

Atopic dermatitis is a commonly chronic pruritic inflammation of the epidermis and dermis. Atopic dermatitis involves cutaneous hypersensitivity. It is characterized by typically distributed eczematous skin lesions of unknown cause. Usually it is accompanied by increased IgE levels in the serum and vascular alterations and slight lymphocytic infiltration (Figure 5). In addition, the number of mastocytes is sometimes increased. Moreover, it has been shown that atopic dermatitis involves IL-4 and IFN-γ release from peripheral blood mononuclear cells (Kaminishi, *et al.* 2002)

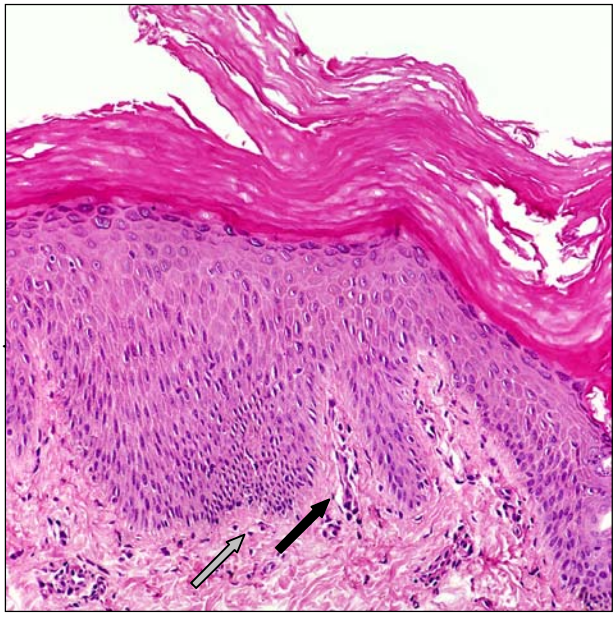


Figure 5: Histology of atopic dermatitis*. Staining of an atopic dermatitis tissue section by hematoxilin/eosin. Blood vessels (black arrow) and infiltrated lymphocytes (gray arrow) are indicated. Magnification: x 200. With permission from: Atlas of dermatology, Feith et al.*

2.5.5 Erythema exudativum

Erythema exudativum (EE) is a skin disease characterized by an eruption of maculae and vesicles. It arises in the course of a reaction to various infections or drugs which induce tissue damage due to humoral and cell mediated immune response with subsequent release of IC. Histologically EE is characterized by an infiltrate of mononuclear cells and neutrophils and by vasodilatation and swelling of the vascular endothelium with moderate erythrocyte extravasation (Figure 6).

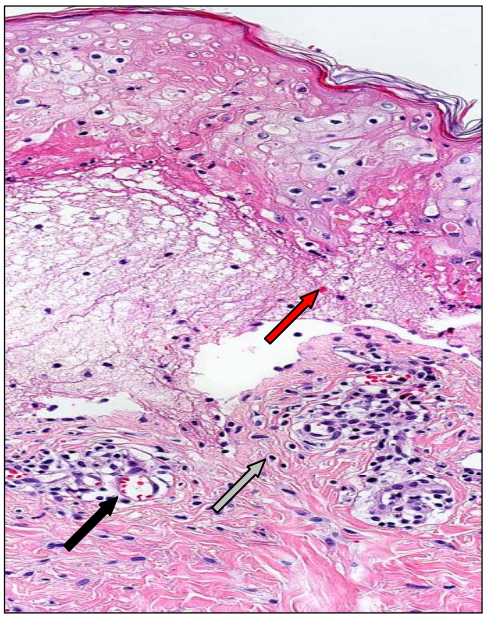


Figure 6: Histology of erythema exudativum multiforme (EEM)*. Staining of an EEM tissue section by hematoxilin/eosin. Dilated blood vessels are indicated by a black arrow. Inflammatory dermal infiltrate (gray arrow) and extravasated erythrocytes (red arrow) are indicated. Magnification: x 200. With permission from: Atlas of dermatology, Feith et al.*

2.5.6 AIDS-associated Kaposi´s Sarcoma

Kaposi's sarcoma (KS) is considered a neoplasm of vascular origin. The most aggressive form, AIDS-associated KS, is associated with infection of two different viruses: human herpes-virus-8 (HHV-8) and human immunodeficiency virus-1 (HIV-1) (Stürzl*, et al.* 2001).
KS lesions evolve histologically through three progressive stages characterized by different histological presentation and cellular composition. (**i**) Early *patch-stage* lesions are flat and are characterized by a network of capillary-like vascular structures of different lumina (Figure 7), extravasion of red blood cells and infiltration of inflammatory cells (T-cells and monocytes/macrophage) (Uccini*, et al.* 1994; Fiorelli*, et al.* 1998; Sirianni*, et al.* 1998). These infiltrated cells lead to increased local concentrations of IC including IFN-γ, IL-1β and TNF-α (Stürzl*, et al.* 1995; Fiorelli*, et al.* 1998; Ensoli*, et al.* 2000; Guenzi*, et al.* 2001; Stürzl*, et al.* 2001). This stage has a granulation-tissue like appearance (McNutt*, et al.* 1983). (**ii**) In the *plaque-stage*, the lesions evolve into thickened papules characterized by increasing numbers of spindle-shaped cells, called "KS spindle cells", and numerous thin-walled vessels filled with erythrocytes. The KS spindle cells, considered the tumor cells of KS, are of endothelial origin and are consistently infected with HHV-8 (Stürzl *et al.* 2001, Ensoli and Stürzl 1998). These lesions exhibit an hemangiosarcoma-like appearance. (**iii**) Finally, in the *nodular-stage*, the KS spindle cells are the predominant cell type that lead to a fibrosarcomatous-like histological appearance (Stürzl *et al.* 2001).
It is a peculiarity of AIDS-KS that it appears at onset simultaneously in multiple lesions at several different areas of the body in the absence of metastasis.

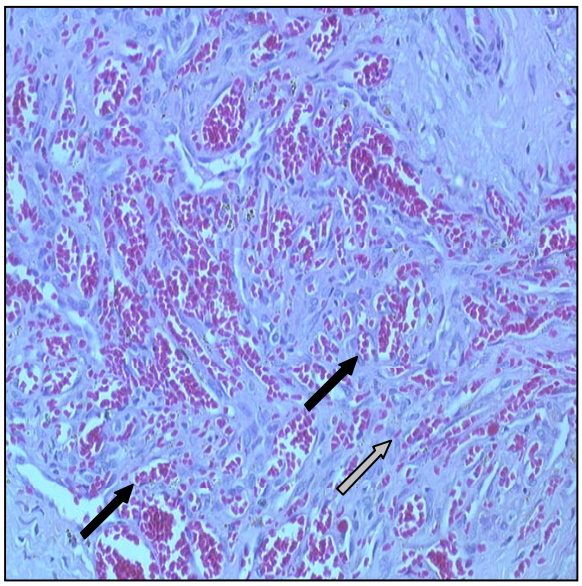


Figure 7: Histology of KS. *Staining of KS tissue section by hematoxilin/eosin. Blood vessels (black arrows) and extravasated of erythrocytes (gray arrow) are indicated. Magnification: x 250.*

There is evidence that, in its early stages, KS may be a hyperplastic, cytokine triggered, reactive process that only in late stages may transform to a real sarcoma (Brooks 1986; Ensoli, *et al.* 2000; Stürzl, *et al.* 2001). KS initiates in a context of immune dysregulation characterised by CD8+ T cell activation and the production of Th1-type cytokines. Infiltrated monocytes are the main source of IC in KS lesions (Stürzl, *et al.* 1995; Fiorelli, *et al.* 1998; Ensoli, *et al.* 2000; Guenzi, *et al.* 2001; Stürzl, *et al.* 2001). IC induce a generalised activation of EC leading to adhesion and tissue extravasation of lympho-monocytes, spindle cell formation and angiogenesis. In addition, inoculation of IC in nude mice induces the formation of KS-like lesions indicating that inflammatory cytokines can trigger the cascade of events leading to KS lesion initiation (Samaniego, *et al.* 1995). IFN-γ, IL-1β and TNF-α are found in chronically elevated concentrations in the serum and also in the tissues of AIDS-KS patients (Hober, *et al.* 1989; Emilie, *et al.* 1990; Vyakarnam, *et al.* 1991; Stürzl, *et al.* 1995; Ensoli, *et al.* 1998; Ensoli, *et al.* 2000; Stürzl, *et al.* 2001)

In every stage a prominent vascularization is observed in KS lesions, which is comparable to the angiogenic process occurring during the growth of solid tumors (Salahuddin, *et al.* 1988). In fact, it has been shown that the KS spindle cells expressed and released bFGF and VEGF *in vitro* and *in vivo* and cell culture supernatants of KS spindle cells have been shown to induce the formation of KS-like lesions in mice (Salahuddin, *et al.* 1988; Thompson, *et al.* 1991; Ensoli, *et al.* 1994; Cornali, *et al.* 1996; Samaniego, *et al.* 1995; Samaniego, *et al.* 1998). Of note, expression of both factors was significantly increased in the late stage lesions as compared to the early stage lesions (Stürzl, *et al.* 2001).

3 Molecular markers of endothelial cell activation

Endothelial cell activation in the described diseases can be indirectly followed by the detection of different markers. Each marker has different peculiarities as described below.

Adhesion molecules such as VCAM-1 and ICAM-1 indicate EC activation, but they are only upregulated by IC for a limited time (less then 24 h). Moreover, these adhesion molecules are downregulated by AGF (Swerlick, *et al.* 1992; Haraldsen, *et al.* 1996; Wahbi, *et al.* 1996; Zietz, *et al.* 1996; Kim, *et al.* 2001a; Kim, *et al.* 2001b; Lee, *et al.* 2001; Tilghman, *et al.* 2002). Increased expression of adhesion molecules has been observed during sepsis were endothelial cell activation and damage occur [reviewed in (Reinhart, *et al.* 2002)].

ICAM-1 is also used as a soluble marker to detect activation of EC. Soluble ICAM (sICAM-1) is a marker that indicates IC-activation of EC (Gho, *et al.* 1999). It has been shown that the concentration sICAM-1 is increased in a number of pathological states, including inflammation and sepsis. In particular sICAM-1 correlates with the severity of inflammation in the course of the disease. In addition sICAM shows persistent elevated concentrations over the first week in sepsis patients (Sessler, *et al.* 1995; Ogawa, *et al.* 2000). However, sICAM is upregulated not only by IC, but also by VEGF (Lu, *et al.* 1999; Kim, *et al.* 2001).

Von Willebrand factor (vWF) is also used as a marker suggestive of damage/injury of the endothelium (Blann 1991; McGregor, *et al.* 1994). IL-1β induces secretion of intracellular vWF, but IFN-γ and TNF-α inhibit the release of vWF from EC (Tannenbaum, *et al.* 1990). vWF expression is up-regulated in EC by angiogenic factors like bFGF and VEGF, which act also synergistically (Zanetta, *et al.* 2000). Increased serum concentrations of vWF have been detected in patients with sepsis (Wanecek, *et al.* 2000; Reinhart, *et al.* 2002).

Thrombospondin (TSP) is a further marker of inflammation. TSP production by EC is decreased by treatment of the cells with IL-1β and TNF-α alone or in combination (Morandi, *et al.* 1994). By contrast, TSP has been shown to be up-regulated by growth factors like epidermal growth factor (EGF) and transforming growth factor-β1 (TGF-β1). TSP secretion has been shown to be up-regulated in glomerulopathies (Okamoto, *et al.* 2002).

Endothelin-1 is secreted constitutively by EC and participates in the regulation of the vascular tone [reviewed by (Wanecek, *et al.* 2000)]. Endothelin-1 secretion is stimulated by IC, endotoxins, and hypoxia (Wang, *et al.* 2002). By contrast endothelin-1 secretion is inhibited by thrombin. Notably, shear stress has opposite effects on endothelin-1 secretion in micro-vascular EC (increase of endothelin-1 secretion; Wang, *et al.* 2002) and in macro-vascular EC

(decrease of endothelin-1 secretion; McCormick*, et al.* 2001). Therefore, changes in endothelin-1 secretion from micro- and macro-vascular EC can compensate each other leading to difficulties in the detection of changes in circulating endothelin-1. The highest concentrations of endothelin-1 in the plasma have been observed in patients with sepsis (Wanecek*, et al.* 2000).

Thy-1 (a cell-surface glycoprotein) has also been discussed as a soluble marker of EC activation (Saalbach*, et al.* 1999). However, not only EC, but also fibroblasts can be the source of soluble Thy-1 (Saalbach*, et al.* 1999). Therefore Thy-1 cannot be used as a activated-EC specific marker.

Also exhaled gas like nitric oxide has been used as a marker of pathologic vasodilatation in sepsis (Stewart*, et al.* 1995).

Finally, a marker commonly used in histology to study cell proliferation is Ki67. Anti-Ki67 antibodies rect with a human nuclear cell proliferation-associated antigen that is expressed in all active parts of the cell cycle. This nuclear antigen is a well established marker to detect proliferating cells in microwave-processed formalin-fixed paraffin sections (Cattoretti*, et al.* 1992). However, Ki67 is not specific for endothelial cells.

3.1 Complexity and redundancy of endothelial cell activation

In all of the diseases described above, activation of EC is regulated by a complex network of different stimuli like interaction with blood cells, with allergenic substances and with soluble factors like IC and AGF originating from the blood or from neighbouring cells. Dependent of the activation, EC adapt their function and morphology to the specific requirements in the tissue. Such changes can involve proliferation (during angiogenesis), apoptosis, invasion (sprouting of vessels during wound healing), migration, adhesiveness towards leukocytes (wound healing, atherosclerosis) and secretion of soluble factors.

Due to the fact that many factors are involved and that the number of different activations is limited, it is likely that different factors can lead to the same phenotype of activated EC. These factors may be put together in groups with functional homology. For example the two AGF, VEGF and bFGF are potent inducers of EC proliferation, while IC like IFN-γ, IL-1β and TNF-α are all potent inhibitors of EC proliferation and induce leukocyte adhesion.

At present, the spatial and temporal response of EC to these different stimuli in tissues is only poorly characterized. Presently it is not possible to determine when and were different groups of factors act on a single EC in tissues. Moreover, the relations between different activation

states of EC are unknown as yet. For example it has to be determined if all activation phenotypes can be present at the same time in one cell or if, due to cell-biological restrictions, the different activation phenotypes are appearing in a temporally or spatially separated manner.

Recent data support the hypothesis of a sequential activation of endothelial cell during pathological processes. For example in tumor angiogenesis or in rheumatoid arthritis it has been shown that the recruitment of leukocytes precedes the formation of blood vessels (Folkman 1995).

3.2 The Guanylate Binding Protein-1: a molecular marker of inflammatory cytokine activated endothelial cells

In order to isolate molecular markers that may characterize IC activated EC in tissues this laboratory analyzed the gene expression of EC in the presence of IC and AGF by differential display RT-PCR (DDRT-PCR). The gene encoding the guanylate binding protein-1 (GBP-1) was the only one of several differently expressed genes identified in the DDRT-PCR study that was upregulated similarly by IL-1ß, TNF-α and IFN-γ at the mRNA level, both in HUVEC and in dMVEC (Guenzi*, et al.* 2001). By contrast, VEGF- or bFGF-treated cells only weakly expressed GBP-1 mRNA (Guenzi*, et al.* 2001). Of note, IC-induced GBP-1 mRNA expression was consistently reduced by the simultaneous addition of AGF (Guenzi*, et al.* 2001). Therefore, GBP-1 may be a molecular marker for IC activated EC. Subsequent studies showed that GBP-1 mediates the antiproliferative effect of IC on endothelial cells without affecting cell adhesiveness (Guenzi*, et al.* 2001).

GBP-1 belongs to the family of large GTPases. Large GTPases are GTPases of high molecular weight that do not need guanine nucleotide exchange factors or GTPase activating proteins (GAP) in order to hydrolyse GTP. The unique position of GBP-1 amongst known GTPases is further demonstrated by its ability to hydrolyse GTP to GDP and GMP with subsequent cleavage of orthophosphate; GDP alone cannot serve as a substrate for GBP-1 (Schwemmle*, et al.* 1994; Neun*, et al.* 1996; Praefke*, et al.* 1999). In addition, GBP-1 binds the guanine nucleotides with weak affinity and has a high turnover GTPase activity that has been explained by the involvement of a GAP domain in GBP-1 (Prakash*, et al.* 2000b). GBP-1 has also been shown to oligomerize *in vitro* (Praefke*, et al.* 1999, Prakash*, et al.* 2000a)

In humans at least five isoforms of GBP are known (summarized in Table 1). All GBPs have at least two motifs of the three classical guanylate-binding motifs, GXXXXGKS (T) and

DXXG, they bind GMP in addition to GDP and GTP. Whereas there is little primary sequence homology to the other large GTPases like dynamin the relationship between the proteins becomes evident by the common architecture of the protein domains and by common biochemical features like nucleotide-dependent oligomerisation and cooperative GTPase activity (Prakash, *et al.* 2000b).

Table 1: Homologies between GBP-1 and the other members of the family of the large GTPases

Isoform	AA %	Nucleotide %	*Gene Bank*	Reference
GBP-1	100	100	M55542	(Cheng, *et al.* 1983; Saunders, *et al.* 1999)
GBP-2	76	82	M55543	(Nguyen et al. 2002; Neun *at al*. 1996)
GBP-3	22	*N.s.*	AF444143	(Luan, *et al.* 2002);
GBP-4	50	80	NM_052941.1	(Nguyen, *et al.* 2002)
GBP-5	65	78	NM_052942.1	
MxA	*N.s.*	*N.s.*	M30817	(Aebi, *et al.* 1989)
Dynamin	*N.s.*	*N.s.*	L36983	(Diatloff-Zito, *et al.* 1995)
DLP1 (rat)	*N.s.*	*N.s.*	L36983	(Yoon, *et al.* 1998)

AA: percentage of homology comparing amino-acid sequences. DLP1 = dynamin like protein
Nucleotide: percentage of homology comparing cDNA sequences. N.s. = not significant

Other members of the large GTPase protein family are Mx (Aebi, *et al.* 1989) proteins and dynamin (Diatloff-Zito, *et al.* 1995) (Table 1). These large GTPases have a similar domain composition and GTPase activity, but sequence homology is very low (Table 1) (Prakash, *et al.* 2000a).

Mx proteins are interferon-induced GTPases that accumulate in the cytoplasm of interferon-treated cells, partly associating with the endoplasmic reticulum. A unique property of Mx GTPases is their antiviral activity against a wide range of RNA viruses [reviewed in (Haller, *et al.* 2002), see also (Accola, *et al.* 2002)]. Mx proteins have two functional domains, an N-terminal GTP-binding domain and a C-terminal effector region involved in self-assembly and viral target recognition (Haller, *et al.* 2002).

Mx proteins are key components of the interferon-induced antiviral state against RNA viruses; in particular, Mx proteins interfere with the intracellular transport of viral components. In the case of bunyaviruses, that have a cytoplasmic replication phase, MxA interferes with transport of the viral nucleocapsid protein (N) to the Golgi compartment, the site of virus assembly. Association of MxA to the viral nucleocapsid protein leads to the sequestration of the viral protein into highly ordered perinuclear complexes and, as a consequence, to the inhibition of viral replication and formation of progeny viruses (Haller, *et*

al. 2002, Accola*, et al.* 2002). In the case of orthomyxoviruses, MxA prevents the incoming viral nucleocapsids from being transported into the nucleus, the site of viral transcription by interacting with the ribonucleoprotein complex of the virus (Haller*, et al.* 2002, Accola*, et al.* 2002).

Dynamin is a large molecular weight GTPase that assembles into oligomers, forming rings or spirals. It can self-assemble or assemble on other macromolecular structures that result in an increase in its GTPase activity (Eccleston*, et al.* 2002). Dynamin is involved in clathrin-dependent endocytosis, even the role of dynamin in vesicle formation remains controversial as to whether it behaves as a mechanochemical enzyme or as a molecular switch [reviewed in (Danino*, et al.* 2001; Thompson*, et al.* 2001; Eccleston*, et al.* 2002)]. In addition, dynamin has been involved in other intracellular trafficking events including exocytosis where it has been proposed to act as a "pinchase" at the trans-Golgi surface to liberate nascent, clathrin-coated secretory vesicles; dynamin has also been implicated in the regulation and maintenance of cell shape [reviewd in (Eccleston*, et al.* 2002)]

As a typical member of the large GTPases, the crystal structure of GBP-1 can be divided into two domains with similar size (Figure 8): (**i**) one compact α-β domain at the N-terminus which contains the GTPase domain (Figure 8, pink) and (**ii**) a long, purely α-helical domain (Figure 3, blue-green) (Prakash*, et al.* 2000a; Prakash*, et al.* 2000b). Moreover, GBP-1 has a potential CAAX isoprenylation motif at its C-terminal end (Figure 3, CAAX) (Asundi*, et al.* 1994; Nantais*, et al.* 1996). In the course of isoprenylation an isoprenyltransferase transfers an isoprenyl group to the sulphur atom in the cysteine of the CAAX motif and the terminal phosphates of the isoprenyl group are removed. Then a protease removes the terminal three amino acids. Finally a carboxy methyltransferase methylates the new exposed C-terminal (Dai*, et al.* 1998; Choy*, et al.* 1999). Isoprenylation is a post-translational modification of proteins that increases the hydrophobicity of proteins and therefore often regulates membrane association (Fu*, et al.* 1999; Hofemeister*, et al.* 2000).

Biological functions of GBPs, except for GBP-1, are not known yet. GBP-1 was originally discovered as one of the major IFN-γ-induced factors (Cheng*, et al.* 1983; Cheng*, et al.* 1985; Boehm*, et al.* 1998). It has been shown that GBP-1 mediates the IFN-γ antiviral effects against the vesicular stomatitis virus and the encephalomyocarditis virus in HeLa cells with an unknown mechanisms (Anderson*, et al.* 1999). Moreover, this laboratory showed that GBP-1 is necessary and sufficient to mediate the anti-proliferative effect of IC on EC. Structure-function analysis of the GBP-1 molecule demonstrated that the isoprenylation of the protein at the C-terminal end as well as the GTPase activity and as the whole globular domain (Figure 8) are not required for the

inhibition of cell proliferation (Guenzi, *et al.* 2001). In contrast, expression of the C-terminal helical domain (Figure 8) inhibited AGF-induced proliferation at a similar extent as the wild type GBP-1. In addition, inhibition of GBP-1 expression abrogated the inhibitory effect of IC on EC. Therefore, the helical domain is the mediator of the GBP-1 antiproliferative activity (Guenzi, *et al.* 2001). The inhibition of cell proliferation by GBP-1 occurs in absence of apoptosis and does not affect IC-induced adhesiveness of monocytes on EC (Guenzi, *et al.* 2001).

These finding indicated that GBP-1 may be an important marker and regulator of the IC-activated phenotype of EC.

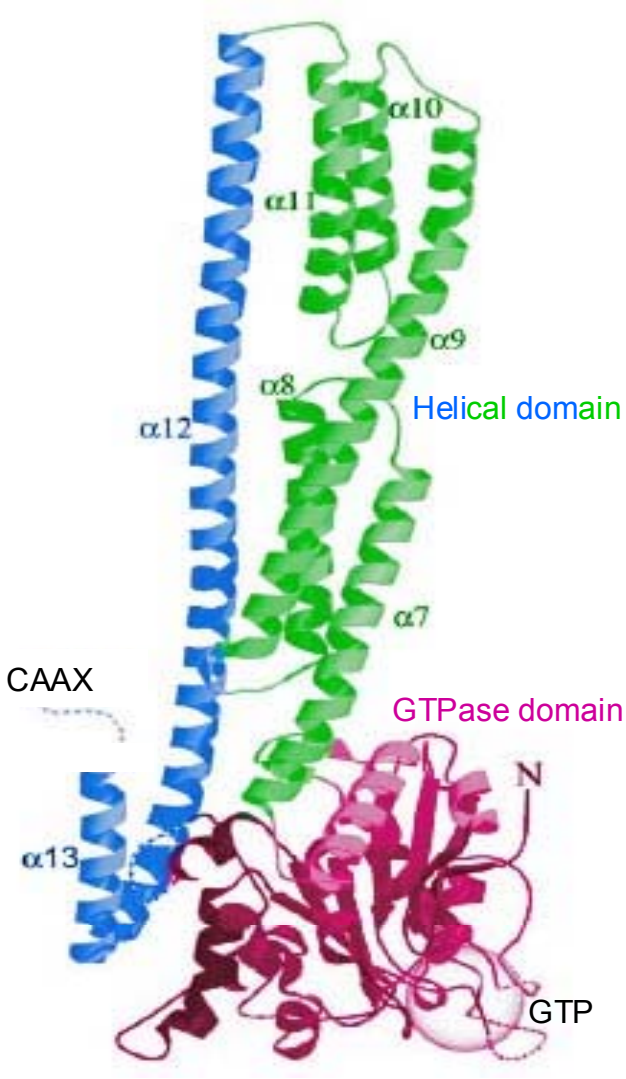


Figure 8: Crystal structure of GBP-1. *GBP-1 protein can be divided into two domains of about the same size. A compact globular α-β domain, containing the GTPase activity at the N-terminus (pink, pink circle represents GTP). A long, purely α- helical domain (green and blue). Moreover, GBP-1 has a potential isoprenylation motif (CAAX). Adapted from (Prakash, et al. 2000a)*

4. Goals of the project

Until now GBP-1 expression has been investigated only at mRNA level *in vitro*. Previous studies on GBP-1 mRNA indicated that it may be a molecular marker of the IC-activated phenotype of EC. IC-activated EC play a key role in inflammatory diseases. GBP-1 expression at the protein level *in vitro* has not been investigated so far. Also *in vivo* the expression of GBP-1 in healthy and/or diseased human tissues has not been investigated. Therefore the aims of this work were first, to investigate whether GBP-1 may be a molecular marker of IC-activated EC at the protein level *in vitro*. Second, to investigate in which cells GBP-1 is expressed in human healthy and/or diseased tissues. Third, to investigate whether GBP-1 may be a molecular marker of the IC-activated EC *in vivo*. Specific goals of the work were:

(1) to purify recombinant GBP-1,

(2) to generate mono- and poly-clonal antibodies against GBP-1,

(3) to investigate GBP-1 expression and subcellular localization at the protein level in different cell types under different stimuli *in vitro*,

(4) to investigate GBP-1 protein expression in different human tissues and in different inflammatory skin diseases,

(5) to investigate if GBP-1 may be secreted,

(6) to develop a sandwich ELISA for the detection of GBP-1 in solution,

(7) to detect circulating GBP-1 in the serum of patients with inflammatory diseases.

MATERIALS AND METHODS

1 Materials

1.1. Chemical reagents

Acetic acid	MERCK
Acrylamid/N,N´Methylenbisacrylamid	BIORAD
Agarose	FMC BIOPRODUCTS
Ammoniumpersulfat	SIGMA
Brefeldin A	SIGMA
Cicloheximide	SIGMA
Coomassie *Brilliant –Blue* (R-250 staining solution)	BIORAD
4',6-diamino-2-phenylindole (DAPI)	MOLECULAR PROBES
D,l-Dithiothreitol (DTT)	SIGMA
Dimethylsulfoxid (DMSO)	SIGMA
EDTA	EDTA
Ethidium bromide	ROTH
Gelatine	SIGMA
Glutamine	GIBCO
Imidazole	SIGMA
Isoperpyl-β-D-thiogalaktopyranosid (IPTG)	BIORAD
Monensin	SIGMA
Methylamine	SIGMA
NiNTA Agarose	QIAGEN
Protein A/G agarose beads	ONCOGENE
Protein standard	AMERSHAM PHARMACIA
Tris(2-carboxyethyl)phosphine (TCEP)	SIGMA
Triton-X100	SIGMA
Trypton	DIFCO
Tween 20	MERCK
Verapamil	SIGMA

1.2 Other solutions

Antibody diluent with background reducing component	DAKO
3,3'-diaminobenzidine (DAB)	BIOGENEX
DNA loading buffer	MBI FERMENTAS
Immumount	SHANDON
Laemmli loading buffer	BIORAD
Gill®-3 Haematoxylin	SHANDON
Percoll	AMERSHAM PHARMACIA
PNPP	ZYMED
RIPA	BOEHRINGER MANNHEIM
Syper-Orange	MOLECULAR PROBES
Trypsin / EDTA solution	NUNC
Vector Red	LINARIS
Western blocking reagent	BOEHRINGER MANNHEIM
Yeast extract	DIFCO

1.3 Olingonucleotides

Sequence	Application
5'-G**GGATCC**G**GAATTC**CTGCATCAGAGATCCACATG-3'	GBP-1 cloning (F)
5'-CT**AGATCTGAGCTC**GCTTATGGTACATGCCTTTCG-3'	GBP-1 cloning (R)
5'-TTCACCGCAGGAAA**A**CTTGCCCAGCTCGA-3'	GBP-1 mutagenesis (F)
5'-TCGAGCTGGGCAAG**T**TTCCTGCGGTGAACG-3'	GBP-1 mutagenesis (R)
5'-ATGGCATCAGAGATCCACATG-3'	GBP-1 sequencing (F)
5'-TTAGCTTATGGTACATGCCTTTCG-3'	GBP-1 sequencing (R)
5'-CCAACTGTACTATGTGACAGAG-3'	GBP-1 sequencing (F)
5'-CCTGTATCCCCTTCCTCGGTTCC-3'	GBP-1 sequencing (R)
5'-TTGAAACAACTGACTGAGAAGA-3'	GBP-1 sequencing (F)
5'-GAGAGAAGCCCTTTTTCTTTCC-3'	GBP-1 sequencing (R)

Bold letters indicate cloning sites in the primers for GBP-1 cloning and the mutated base in the primers used for GBP-1 mutagenesis.

1.4 Enzymes and reagents for molecular biology

Desoxynucleotidetriphosphates (dNTP)	PERKIN ELMER
DNA standard	BOEHRINGER MANNHEIM
Ethidium bromid	ROTH
Restriction enzymes	ROCHE
rTth DNA polymerase	PERKIN ELMER
DNA molecular weight standards	MBI FERMENTAS

1.5 Kits

DC assay	BIORAD
DNA isolation kit	QIAGEN
ECL-detectionsystem	AMERSHAM PHARMACIA
LD-L activity assay	SIGMA
LIVE/DEAD® Cell Viability Kit	MOLECUAR PROBES
Gel purification kit	QIAGEN
QIAquick PCR purification kit	QIAGEN
Rapid DNA ligation kit	ROCHE
Silver Quest	INVITROGEN
Site-directed-mutagenesis kit (Quick-Change)	STRATAGENE
Vectastain Elite ABC	VECTOR LABORATORIES

1.6 Media and supplements

Ampicillin	GIBCO
Bovine skin gelatine, fraction V	SIGMA
Dulbecco's modified Eagle medium (DMEM)	GIBCO
Endothelial cell basal medium (EBM-2)	CLONETICS
Endothelial Cell Growth/Labeling Medium (EGLM-2)	CLONETICS
Fetal bovine serum (FBS)	GIBCO
Glutamine	GIBCO
Kanamycin	INVITROGEN

^{35}S-Methionine	HARTMANN ANALYTIC
Penicillin G	GIBCO
RPMI 1640	GIBCO
Streptomycin sulphate	GIBCO

1.7 Cytokine and growth factors

Angiopoietin-2	R&D SYSTEMS
IFN-α	BIOZOL
IFN-γ	ROCHE
IL-1α	PROMOCELL
IL-1β	ROCHE
IL-4	ROCHE
IL-6	ROCHE
IL-10	ROCHE
IL-18	MEDICAL & BIOLOGICAL L.
IP-10	PROMOCELL
MCP-1	PROMOCELL
MIP-1β	PROMOCELL
Oncostatin M	PROMOCELL
PDGF B/B	ROCHE
PF4	PROMOCELL
SDF-1α	PROMOCELL
TNF-α	ROCHE
VEGF	R&D SYSTEMS
bFGF	ROCHE

1.8 Vectors

PCR-Script	STRATAGENE
pQE 60	QIAGEN
pQE9	QIAGEN
pGEX	AMERSHAM

1.9 Bacterial strains

Name	*Genotype*	
E. coli M15	F^-, Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, $RecA^+$, Uvr^+, Lon^+.	QIAGEN
E. coli XL-1Blue	F':Tn10 perA+B+ laclq D(lacZ) M15/RecA1 end/A1 gyr 96 (Nalr)Thi hsdR17 (rk-mk+) supE44	STRATAGENE
E. coli DH5α	F- *en*dA1 *hs*dR17 (rk -, mk +) *su*pE44 *th*i-1 *gy*rA96 *re*lA1 80*lac*Z M15/RecA1.*(lac*ZYA-*ar*gF) U169	QIAGEN

1.10 Eukaryotic cells
All cell types were from human origin

Name	*Description*	
dMVEC	Primary dermal microvascular endothelial cells	CLONETICS
Fibroblasts	Primary dermal fibroblasts	*Michael Stürzl-GSF*
HaCaT	Normal keratinocyte cell line	*Michael Stürzl-GSF*
HuT 78	Cutaneous T-lymphocyte cell line, lymphoma	*ATCC # TIB-161*
HUVEC	Primary umbilical vein endothelial cells	CLONETICS
JURKAT	T-lymphocyte cell line, acute T-cell leukemia	*ATCC # TIB-152*
Schlicht	B-cell line	*Judith Johnson-LMU*
THP-1	Monocytic cell line, acute monocytic leukemia	*ATCC # TIB-202*
Michl	B-cell line	*Judith Johnson-LMU*
U937	Monocytic cell line, histiocytic lymphoma	*ATCC # CRL-1593.2*

1.11 Paraffin-embedded Tissues

Multi-tissue control slides	BIOGENEX
AIDS-associated Kaposi's sarcoma	DEPT.DERMATOL., ESSEN
Pathological tissue	INST. OF PATHOLOGY, LMU
Skin diseases	DEPT. DERMATOL.,VIENNA

1.12 Blood samples

Sera and plasma from healthy people	*Volunteer donors*
Plasma from melanoma patients	RUHR-UNIVERSITY, BOCHUM
Plasma from HIV-infected people	RUHR-UNIVERSITY, BOCHUM
Sera from people with inflammatory skin diseases	DEPT. DERMATOL.,VIENNA

1.13 Antibodies and lectins

β-actin	SIGMA
Alexa Fluor conjugates	MOLECULAR PROBES
Anti rabbit-AP	ZYMED
AP-1 (c-jun)	ONCOGENE
Cathepsin-D	R&D
Caveolin1	R&D
CD3	DAKO
CD8	SEROTEC
CD31	SEROTEC
CD34	SEROTEC
CD68	DAKO
Concanavalin A-Alexa594	MOLECULAR PROBES
Fab anti-mouse	DIANOVA
GAPDH	CHEMICON
Ki67	DAKO
Lamp-1	R&D
MMP-1	R&D
PCNA	R&D
Normal goat serum	DIANOVA
Phospho-caveolin	R&D
Rab5	R&D
Rab7	SANTA CRUZ
Sec23	SANTA CRUZ

Secondary antibodies coupled to Alexa fluorochromes	MOLECULAR PROBES
TGN38	R&D
αTubulin	MOLECULAR PROBES
VCAM-1	SANTA CRUZ

1.14 Columns

MT5	BIORAD
Disposable columns (EconoPac)	BIORAD
Desalting columns (HiTrap deasalting)	AMERSHAM PHARMACIA

1.15 Equipment

Blotting apparatus	AMERSHAM PHARMACIA
CCD camera	SONY
Electroporation apparatus	BIORAD
ELISA plates (NUNC-immunoplates)	NUNC
ELISA reader	BIORAD
Filters-(45 μm)	MILLIPORE
French-press apparatus	SLM AMINCO
FPLC device (BioLogic)	BIORAD INVERSE
Hybond –P membran	AMERSHAM PHARMACIA
Hyperfilm	AMERSHAM PHARMACIA
Light scanning microscope (LSM 510)	ZEISS
Imagequant (Documentation system for gels)	BIORAD
MICROSCOPE/ epifluorescence	LEIZ
PCR thermal cycler GeneAmp 2400	PE APPLIED SYSTEMS
pH meter	HANNA INSTRUMENTS
Protein fraction collector	BIORAD
Spectrophotometer	CECIL
Spectrophotometer (Gene Quant II)	AMERSHAM PHARMACIA
Filters (Centricon)	AMICON
Water baths	JULABO
Weighing machines	METMER
X-ray film developing machine	AGFA

1.16 Other material

Boyden chambers	
Chambered cover glasses	NUNC
Centricons	AMINCON
Dialysis cassettes	PIERCE
8-weel chamber slides	BECTON DICKINSON
Permanox slides	NALGE-NUNC
Nunc Tubes	NUNC
Immumount	SHANDON
Tissue culture flasks	NUNC

1.17 Centrifuges and rotors

Sorvall RC-5B	DUPONT INSTRUMENTS
Sorvall RC-5C	DUPONT INSTRUMENTS
Beckmann J2-21M/E	BECKMANN INSTRUMENTS

1.18 Computer programmes

Corel PhotoPaint 8	COREL CORPORATION
EndNote4	ISI RESEARCH SOFT
EXCEL	MICROSOFT
Illustrator 9.0	ADOBE
Image Quant 1.2	MOLECELAR DYNAMICS
LSM5 Image Browser	ZEISS
Photoshop 5.0	ADOBE
SPSS 8.0	SPSS ADVANCED STATISTICS
Windows NT	MICROSOFT

1.19 Buffers

TAE containing (0.4 M tris base, 0.2 M acetic acid, 0.1 M EDTA; pH 8.4)

PBS containing (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2PO_4, 1.4 mM K_2PO_4; pH 7.4)

2 Methods

2.1 Cell biological methods

2.1.1 Mammalian cell culture

All cells were cultivated under aseptic conditions in tissue culture flasks at 37 °C in the presence of 5 % CO_2. Growth medium was changed every 2-3 days.

Adherent HeLa cells, human primary fibroblasts and the keratinocyte cell line HaCaT were grown with Dulbecco modified Eagle´s medium (DMEM) and supplemented with 10 % FBS, 2 mM L-glutamine, 50 U/ml penicillin G and 50 µg/ml streptomycin sulphate. Both HUVEC and dMVEC were grown in complete endothelial cell basal medium (EBM-2) supplemented with 5 % FBS and propagated in cell culture flasks coated with bovine skin gelatin, type B, 1.5 % in PBS. The T cell lines HuT 78 and Jurkat, the B-cell lines Schlicht and Michl and the monocytic cell lines U937 and THP-1 were grown in RPMI 1640 medium supplemented with 10 % FBS, 50 U/ml penicillin G and 50 µg/ml streptomycin sulphate.

The cells were split into new culture flasks when they reached 80-90 % confluence. Old medium was removed and the cells were washed twice with sterile PBS buffer. Then 2 ml of trypsin / EDTA solution (0.025 % trypsin/0.01 % EDTA: 1x, stock solution of 10x diluted in PBS) was added. Trypsin/EDTA in excess was discard and the culture flask was incubated at RT and observed under the microscope until cells detached from the surface of the flask. Then 10 ml of fresh medium containing 10 % serum was added to inactivate the activity of trypsin.

Cells were centrifuged (except for primary cells) at 400 *g* for 5 min at RT. Medium was discarded and the cells were resuspended in fresh growth medium.

2.1.2 Cell stimulation with different factors

For cytokine induction cells were maintained ON (16 h) in the appropriate medium with 0.5 % FBS (low medium, except for PBMCs) in order to synchronise a little the cell cultures. The factors were diluted to the stock concentrations recommended by the manufacture in BSA 0.1 % in PBS. For further use the cytokines were diluted directly in the cell culture medium (low medium, if not otherwise specified). The tips were coated with BSA 0.1 %. For stimulation of the cells the needed factor was added in fresh low medium at the indicated concentrations and times. Usually as a control cells were stimulated with an equal volume of BSA 0.1 % only.

2.1.3 Inhibition of secretory pathways

For inhibition of different secretory pathways, drugs were added to the cell cultures before IFN-γ stimulation at the indicated times: monensin (1 nm, 1 h), methylamine (1 nm, 1 h), verapamil (500 ng/ml, 30 minutes) and BFA (1 µg/ml, 2 h). The drugs were resuspended in water or ethanol, cells were stimulated in equal volume of ethanol as a control.

2.1.4 Freezing of cells

Cells were harvested from culture flasks as described above (**2.1.1**). After centrifugation (400 *g*, 10 min, RT), the medium was removed. Cells were then resuspended in ice-cold freezing medium [the appropriate growing medium supplemented with 20 % (v/v) serum and 10 % (v/v) DMSO] at a concentration of 10^6 / 10^7 cells/ml. Afterwards, 1 ml aliquots of cell suspension were dispensed into criotubes (1.8 ml). The tubes were placed into wells of a brass block pre-cooled at 4 °C. The block was then kept at –70 °C for 24 h after which the ampoules of cells were transferred to liquid nitrogen for long-term storage.

For revival of cells a frozen ampoule was thawed rapidly in a 37 °C water bath, disinfected and the content was put in a cell culture flask with pre-warmed medium. After 6 hours the medium was discarded and fresh pre-warmed medium was added.

2.1.5 Proliferation assay

Proliferation assay was performed on 24 wells or 6 wells culture dishes. Cells were seeded in the appropriate medium in the appropriate number (10^3 - 10^4 cells per well) and let grow ON.

Cells were plated in triplicate and the cell number in each well was counted twice. Cells were washed in low medium and stimulated with the described factors. For long time experiments, the medium was changed at day 2 or 3 and fresh factors were added. At day 4 or 6 cells were splitted, counted. The results were expressed as the mean of the cell number (± SD).

2.1.6 Chemotaxis assay

Chemotaxis experiments were performed with HUVEC using Boyden chambers. Polycarbonate filters of 8 μm pore size were coated with 1.5 % bovine skin gelatine. Low medium (0.5 % FBS) with or without the different factors in the indicated concentrations was placed in the lower compartment of the chamber. Then 2 x 10^4 cells resuspended in low medium were added to the upper compartment. After 2 - 4 h incubation at 37 °C the filters were harvested and the cells were removed from the upper side. Transmigrated cells at the lower side of the filter were fixed with methanol at –20 °C for 4 minutes and stained with Gill-3 Haematoxylin. The number of migrated cells was determined under the microscope. The results were expressed as the mean number (± SD) of migrated cells in five microscopic fields (25 x magnification). Each experiment was performed in triplicate.

2.1.7 Metabolic labeling of cells

The medium from actively growing cells was aspirated and cells were washed twice with PBS. Cells were incubated in EGLM-2 medium (without methionine) for 1 h. Afterwards cells were labelled with ^{35}S-Methionine (27.8-50 μCi/ml) for the desired time.

2.1.8 Determination of cell viability

Cell viability was evaluated by measurement of lactate dehydrogenase (LDH) activity in cell culture supernatants. A commercial assay was employed as recommended by the manufacturer. Briefly, the assay is based on a redox reaction between lactate and nicotine adenin dinucelotide (NAD) to get pyruvate and NADH. The reaction is monitored by spectrophotometric analysis. The absorbance is measured at 340 nm. To correlate the result of the LDH activity assay with cell viability a standard was used: HUVEC were lysed by three cycles of freezing and thawing. Aliquots corresponding to 1 %, 5 %, 7.5 %, 10 % and 20 % or more of the cell number used in the experiment were added to prewarmed medium (37 °C). LDH activity of these standards was measured together with LDH activity of fresh cell culture supernatants of the experiment.

Cell viability was also tested using the LIVE/DEAD® cell viability kit according to manufacturers instructions. The number of dead cells/optical field was compared to the total number of cells/optical field.

2.2 Molecular biological methods

2.2.1 Preparation of plasmid DNA

Preparation of plasmid DNA from *E. coli* was carried out with the Mini-kit purchased from Qiagen. Briefly bacteria were cultivated in 10 ml of LB medium containing 50 µg/ml ampicillin ON at 37 °C with agitation. The cells were harvested by centrifugation (4,000 *g*, 5 min) and plasmids were extracted according to manufacturer's instructions. Plasmids were eluted by incubation of the columns with 30 µl bidest water at RT.

2.2.2 Restriction digest

Digestion of DNA with restriction enzymes was performed according to the manufacturer's instructions using recommended buffer systems and at the appropriate reaction temperatures. Generally, 1 U of enzyme was needed per µg DNA. Plasmid DNA was usually digested for 1-2 h. The completion of the reaction was monitored by agarose gel electrophoresis.

2.2.3 Agarose gel electrophoresis

Electrophoretic separation of DNA was carried out using 0.7-1.5 % (w/v) agarose gels containing ethidium bromide (5 µg/ml) in 1x TAE buffer. The gel buffer and running buffer were identical. Gels were cast in chambers of various sizes, and the DNA was mixed with DNA sample buffer and loaded onto the gels. Separation occurred at 1-5 V/cm. Subsequently gels were observed under UV light at 312 nm and pictures were taken with the gel documentation system.

2.2.4 Isolation of DNA fragments from agarose gels

Extraction and purification of DNA from agarose gels in TAE buffer were performed using the QIAquick gel extraction kit from Qiagen according to the manufacturer's instructions. Buffers were provided by the kit and all centrifugation steps were carried out at 10,000 g at RT in a table-top micro-centrifuge. DNA elution was performed by the addition of 30 µl of bidest water, incubation at RT for 1 h followed by centrifuging for 1 min. If needed, DNA was concentrated by precipitating with 2 vol. ethanol.

2.2.5 Oligonucleotide primers

All oligonucleotide primers were synthesized from the GSF service and delivered as lyophilised forms. The oligonucleotides were dissolved in 100 µl of sterile water. Oligonucleotide concentration was determined by measuring the optical density (OD) at 260 nm. Oligonucleotide primers used for GBP-1 cloning (bold letters show the restriction sites), for site directed mutagenesis (bold letters indicate the mutated bases) and for sequencing are listed in the section Materials (paragraph **1.3**):

2.2.6 Polymerase chain reaction (PCR)

The coding region of GBP-1 (*GeneBank* accession number M55542) was amplified by PCR from the full length GBP-1 cDNA cloned into the pCR-Script vector available in the laboratory. The reaction was performed in 50 µl volume (0.5 µg plasmid DNA, 8 µl dNTP mix, 0.3 µM of each primer, 4 U *rTth* DNA polymerase, 7 µM $MgCl_2OAc$, 5 µl 10X PCR buffer) and overlaid with mineral oil at the surface to avoid evaporation.

The PCR amplification of GBP-1 was performed with following protocol:

Step	*Temperature*	*Time*	*N° of cycles*
Initial denaturation	96°C	2 min	1
Denaturation	94°C	20 sec	5
Annealing	52°C	30 sec	
Elongation	68°C	3 min	
Denaturation	94°C	20 sec	20
Final elongation	72°C	10 min	
	4°C	∝	1

2.2.7 Purification of PCR products

PCR products were purified using the QIAquick PCR purification kit following the manufacturer's protocol. Buffers used were provided by the kit and all centrifugation steps were performed at 10,000 *g* at RT using a table-top micro-centrifuge. DNA was eluted by the addition of 30 µl of bidest water.

2.2.8 DNA ligation

DNA fragments were ligated using the rapid DNA ligation kit from Roche according to manufacturer's instruction with a particular attention to the molar ratio of the vector and insert DNA fragment. Cohesive-end ligation was carried out at RT for the suggested 4 min.

2.2.9 Site-directed mutagenesis

Site directed mutagenesis was performed within the GBP-1 coding sequence (bp 806) of the GBP-1-His construct using the Quick-Change Kit from Qiagen according to manufacture's instruction. Briefly, the nonstrand-displacing *Pfu-Turbo* polymerase was used. The methylated, non mutated parental DNA template was digested with *DpnI*. The sequence of the mutagenic primers were described above (**1.3**). The created G to A transition deleted the GBP-1 gene internal *HindIII* site.

2.2.10 Cloning of GST-GBP1-His

The mutated GBP1-His insert was cut with *HindIII* and *EcoRI* and cloned into the pGEX vector using the techniques described above.

2.2.11 His-GBP-1, His-GBP-2

These clones were a gift of Martin Schwemmle (Universität Freiburg). The two constructs have a 6 x histidine (His) tag in 5' of the GBP-1 sequence. The clones were tested by sequencing.

2.2.12 Preparation of electroporation competent cells

One litre of LB medium was inoculated with 10 ml of an overnight culture of the *E. coli* strain of choice. The large scale culture was incubated at 37 °C with shaking until an OD_{600} of 0.6 was reached. All tubes and solutions used were sterilised and cooled to 4 °C. The cells were cooled on ice, harvested by centrifuging (4,000 *g*, 15 min, 4 °C) and resuspended in 1 l of deionized water. Following another centrifugation step, the cells were resuspended in 0.5 l of deionized water and pelleted again. Then the cells were washed with 20 ml of 10 % glycerol, centrifuged and finally resuspended 3 ml of 10 % glycerol. 200 µl aliquots were frozen on dry ice and stored at –80 °C.

2.2.13 Transformation of electrocompetent cells.

Typically 1 µl of plasmid DNA was added to 100 µl of electrocompetent *E. coli* on ice. Electroshock was performed using a pre-chilled transformation chamber by 1.8 kV. The length of the impulse was typically 5 msec. Transformed cell were than incubated with 900 µl of pre-warmed SOC medium for 1 h and plated on agar plates.

2.2.14 Heat-shock transformation of *E. coli.*

For transformation 100 µl of competent *E. coli* cells were pre-incubated on ice with 1.7 µl β-mercaptoethanol (provided by the manufacturer) for 1 min. Typically 1 µl of plasmid DNA was added and incubation on ice was performed for 30 more minutes. Cells were than incubated for exactly 30 sec at 42 °C in a water bath followed by 2 min incubation on ice. Transformed cell were than incubated with 900 µl of pre-warmed SOC medium for 1 h and plated on agar plates.

2.2.15 Screening for positive *E. coli* transformants

In order to isolate *E. coli* colonies carrying the desired DNA fragment, colonies were picked and mini preparations of plasmid DNA as described above (**2.2.1**) were performed followed by restriction analyses (**2.2.2**) to determine the correct orientation of the DNA insert. Finally the authenticity of the DNA sequence was verified by sequencing. Sequencing was carried out using a commercial service (Sequiserve).

2.2.16 *E. coli* permanent cultures

Important transformants were preserved as permanent cultures. An inoculum of bacteria was concentrated by centrifugation at 400 *g* and resuspended in 1 ml of LB medium containing 20 % glycerol. The cells were frozen and stored at –80 °C.

2.2.17 Determination of induction kinetics

E. coli cultures were grown under different temperatures varying from 25 °C to 37 °C. IPTG was added to different concentrations varying from 100 µM to 1 mM. Growth curves were determined for every culture by measuring the absorbance at 600 nm at constant time intervals. The optimal condition to overexpress the desired recombinant protein was determined by comparing the protein yield in the soluble fraction as described below (**2.3.7**) of all cultures and choosing a condition in which the cultures were still growing.

2.2.18 Determination of protein solubility

The pellet from 2 ml of *E. coli* cultures were resuspended in 2 ml of bidest water and sonicated six times for 10 sec with 10 sec pauses at 200-300 W. Lysed cells were centrifuged at 10,000 *g* for 30 min at 4 °C. The supernatant was collected (soluble fraction). The pellet was resuspended in an equal volume of bidest water (insoluble fraction). Soluble and

insoluble fractions were separated and analyzed by SDS-PAGE and Coomassie Blue staining as described above (**2.3.8**).

2.3 Biochemical methods

2.3.1 Inhibition of protease activity

A commercial available cocktail of protease inhibitors (final concentrations: 0.02 mg/ml pancreas extract, 5 µg/ml pronase, 0.5 µg/ml thermolysin, 3 µg/ml chymotrypsin and 0.33 mg/ml papain) was prepared just before use and employed when needed.

2.3.1 Preparation of cellular extracts using RIPA buffer

Trypsinized cells (**2.1.1**) were washed twice with ice cold PBS, collected by centrifugation at 400 *g* for 3 min at 4 °C. Cells were than resuspended in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % SDS, 0.5 % sodium desoxycholate, 1 % Nonidet P-40 and cocktail of protease inhibitors) to a final concentration of about 10^7-10^8 cells/ml. Cells were incubated on ice for 10 min and centrifuged at 10,000 *g* at 4 °C for 10 min. The supernatant containing total cellular proteins was collected and stored at –20 °C. Total protein concentration in the supernatant was determined as described below (**2.3.4**).

2.3.3 Thaw-lysis of cells

Trypsinized cells (**2.1.1**) were centrifuged and resuspended in ice cold lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and cocktail of protease inhibitors) to a final concentration of about 10^7-10^8 cells/ml. Successively cells were frozen for 5 min in dry ice mixed with ethanol, transferred to a 37 °C water bath and thawed for 5 min. The freeze/thaw cycle was repeated three times. The cell lysate was cooled on ice and centrifuged at 10,000 *g* for 1-3 hours at 4 °C. The supernatant was collected for further analysis.

2.3.4 Triton extraction of cellular proteins

Extraction of intracellular proteins using the detergent Triton-X100 was performed using a protocol adapted from (Volchuk*, et al.* 2000).

Cell monolayers were washed twice with ice cold PBS. Afterwards cells were incubated for 30 min on ice with lysis buffer (1 % Triton-X100, 20 mM HEPES, 100 mM KCl, EDTA 2 mM, 1 mM DDT and cocktail of protease inhibitors). Cells were scraped from the dish by

pipetting the lysate up and down twenty times. The lysate was centrifuged at 10,000 g for 30 min at 4 °C. Proteins of the supernatants (soluble fraction) were precipitated with TCA (**2.3.5**) and resuspended in a suitable volume of Tris pH 8.8 and in an equal volume of Laemmli sample buffer (2X). The pellet (insoluble fraction) was resuspended in Laemmli sample buffer (2X). Usually from a T75 cell culture flask 30 µl of both soluble and insoluble fraction of proteins were obtained.

2.3.5 Precipitation of proteins by TCA

For precipitation of proteins TCA 60 % was added to the sample to a 10 % final concentration. The sample was incubated on ice for 20 min and centrifuged (10,000 *g*, 10 min at 4 °C), the supernatant was discard. The pellet was washed with 300 µl of ice cold acetone and centrifuged at 10,000 *g* for 10 min at 4 °C; if necessary this step was repeated twice. Acetone was discarded, the pellet was let dry and resuspended in an appropriate volume of Tris pH 8.8 and in an equal volume of Laemmli sample buffer (2X) for analysis by SDS-PAGE.

2.3.6 Determination of protein concentration

Protein concentration was determined using the DC (detergent compatible) protein assay from BioRad according to manufacturer's instructions. This assay is based on the differential colour change of an acidic solution of Coomassie Brilliant Blue G-250 when binding to proteins occurs. Standards were prepared by serial dilutions of bovine serum albumin in PBS or in RIPA buffer.

In some cases protein concentration was determined by comparison of serial dilutions of albumin in PBS and the probe by SDS-PAGE and Coomassie staining.

2.3.7 Western blotting

Samples were boiled in Laemmli buffer for 5 min, size-separated by SDS-PAGE 10 % if not other specified, and electrophoretically transferred to a Hybond-P membrane. Blotting was performed using a wet electroblotter at 250 mA for 2 h. The blots were blocked in 1 x Western blocking reagent solution in PBS-T (0.1 % Tween 20 in PBS) overnight at 4 °C. Then the blots were incubated with the indicated primary antibodies for 1 h at RT. The blots were washed and incubated for 45 min with the appropriate secondary antibody coupled to horseradish peroxidase (1:5,000). All antibodies were diluted in 0.5 x Western blocking in

PBS-T. Detection was performed with the ECL Western blotting detection system according to manufacturer's instructions. Films were usually exposed for 30 sec, 2 min and 5 min.

2.3.8 Coomassie Blue staining of proteins

Protein samples were boiled in Laemmli buffer for 5 min and size-separated by SDS-PAGE. The gels were briefly rinsed in water and stained with Coomassie Brilliant Blue for 30-60 min. Gels were de-stained using a 45 % methanol, 5 % acetic acid (v/v) solution until the background of the gel was clear. Gels were dried between two cellophane sheets for documentation.

2.3.9 Syper Orange staining of proteins

Protein samples were boiled in Laemmli buffer for 5 min and size-separated by SDS-PAGE. The gels were briefly rinsed in water and stained with Syper-Orange diluted 1: 5,000 in 7.5 % (v/v) acetic acid for 40 min. Afterwards gels were rinsed in 7.5 % (v/v) acetic acid and photographed using the gel documentation system. This technique can detect up to 500 ng of protein per lane.

2.3.10 Silver staining of proteins

Protein samples were boiled in Laemmli buffer for 5 min and size-separated by SDS-PAGE. The gels were briefly rinsed in water and fixed in 30 % methanol and 10 % acetic acid. Staining of the gels was performed using a commercial available kit (Silver Quest) according to manufacturer's instructions. This technique can detect up to 1 ng of protein per lane

2.3.11 Protein purification through NiTA affinity chromatography

GBP-1-His, His-GBP-2 and His-GBP-1 were purified under native conditions. *E. coli* M15 containing the respective expression plasmids were grown in 500 ml of LB medium supplemented with ampicillin (100 μg/ml) and kanamycin (25 μg/ml) at 37 °C until an A_{600} value of 0.6 was reached. Then IPTG was added to a final concentration of 100 μM for the GBP constructs and of 200 μM for eGFP-His. The cultures were grown for an additional 4 h (for His-GBP-1 and GBP-1-His), 3 h (for eGFP) or 2 h (for His-GBP-2).

Cells were pelleted and resuspended in 5 ml of buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 % glycerol, 2 mM TCEP, 20 mM imidazole, pH 7.4) and lysed with 2 passages at 1,200 psi in a French press apparatus. The cell lysates were centrifuged at 37,000 g for 60 min. Then the supernatants were filtered (45-μm filters) and loaded onto 5ml NiNTA agarose

columns. A FPLC was performed at RT. Proteins were eluted with a 20-500 mM imidazole gradient. The elution pick was at 150 mM for GBPs and around 250 mM imidazole for eGFP-His.

2.3.12 Protein purification of GST-GBP1-His through NiTA affinity chromatography followed by glutathione affinity chromatography

GST-GBP-1-His was purified under native conditions like in **2.3.11** with slight modifications: (i) cultures were grown at 25 °C; (ii) after IPTG addition the culture was grown for an additional 3 h. The purified recombinant GST-GBP-1-His eluted from the NiNTA FPLC was dialyzed in PBS (pH 7.4) ON. The sample was then loaded on a glutathione sepharose column with 2 ml bed volume, washed with 20 ml PBS and eluted with 2 ml of reduced glutathione (four times for a total of 8 ml) at RT.

2.3.13 Dialysis

For buffer exchange of different samples, dialysing tubes with a cut-off of 30 kDa or dialysis cassettes with a cut-off of 10 kDa were used. Typically dialysis was performed in 2 litres of the appropriate ice cold buffer ON at 4 °C, buffer was changed twice.

2.3.14 Desalting

Desalting was performed using desalting columns according to manufacturer's instructions.

2.3.15 Immunoprecipitation

Fresh cell lysates were pre-cleared by incubation with 2 µl of rabbit pre-immunserum and 25 µl protein A/G agarose beads for at least 3 hours on a rocker platform at 4 °C. After pelleting the beads, the supernatant was incubated with 25 µl protein A/G agarose beads and 1 µl of polyclonal rabbit anti GBP-1 serum overnight on a rocker platform at 4 °C. Beads were washed five times in PBS. The beads were then resuspended in 30 µl Laemmli sample buffer (2X) and boiled for 5 minutes. The samples were separated on 10 % SDS-PAGE followed by Western blot analysis or auto radiography.

For immunoprecipitation of GBP-1, GAPDH or mMP-1 from cell culture supernatants, 10 ml medium were immediately put on ice, centrifuged at 400 *g* for 5 min and filtered through a 45 µm filter. If needed a protease inhibitor cocktail was added immediately. From this step pre-clearing and immunoprecipitation of the proteins from the supernatants were performed as described above for the cell lysate using the appropriate antibodies.

2.4 Production of anti GBP-1 antibodies

2.4.1 Monoclonal antibodies

The production of monoclonal antibodies was performed in collaboration with Elisabeth Kremmer, GSF, Munich. Purified recombinant GBP-1 with a his tag at its C-terminal end (GBP-1-His, 50 μg) was injected intraperitoneally (*i.p.*) and subcutaneously (*s.c.*) into LOU/C rats. Two months later a final boost was given *i.p.* and *s.c.* three days before sacrifice. Fusion of P3X63-Ag8.653 myeloma cells with the rat spleen cells was performed according to the standard procedure. Hybridoma supernatants were tested in a solid-phase immuno-assay using GST-GBP1-His fusion protein adsorbed to polystyrene microtiter plates.

2.4.2 Polyclonal antibodies

Polyclonal antibodies against GBP-1 were produced using a commercial available service "Dr. Pineda", Berlin, Germany, by immunizing three rabbits with GBP-1-His (500 μg) and collecting the antiserum on the ninetieth day after immunization. Pre-bleed serum collected at day one was used as a control.

2.5 Immuncyto- and immunohisto-chemistry

2.5.1 Immunohistochemistry on paraffin embedded sections

Paraffin embedded sections were stained using the Vectastain ABC kit as described by the manufacturer. Briefly, paraffin-embedded sections (6 μm) were dewaxed in xylene and rehydrated. After antigen retrieval by microwave boiling (3 times at 7 min, 580 W) in the appropriate buffer, the slides were treated with 7.5 % hydrogen peroxide for 10 min at RT to block endogenous peroxidases. The slides were then incubated for 1 h with the monoclonal antibody of choice at the dilution indicated in the results or with the respective isotype control antibodies. For controls MAb 1B1 (1 μl) was incubated with 80 μg of purified recombinant GBP-1-His for 2 h at RT. Subsequently, the slides were incubated for 30 min with biotinylated secondary antibody, followed by 30 min incubation with the avidin-biotin complex. The reaction was developed using DAB at RT. Nuclei were counterstained with haematoxylin and mounted with Immunomount. Between each step the slides were washed twice in Tris (50 mM, pH 7.4) for 5 min.

For simultaneous detection of GBP-1 and CD68, the detection of GBP-1 was performed using the Vectastain ABC kit as described above. After the development of the colour reaction wit DAB, the slides were washed in water for 10 min and then twice in Tris (50 mM, Brij 0.5%, pH7.4) for 5 min. Subsequently, slides were incubated with a monoclonal mouse anti-CD68 antibody (1:200) and subsequently with a rabbit anti-mouse IgG antibody (1:25) and the APAAP (mouse) complex (1:50). VectorRed was used as a substrate for the colour reaction. Between each step the slides were washed twice in Tris (50 mM, Brij 0.5%, pH 7.4) for 5 min. After the immunohistochemical procedure, sections were counter-stained with hematoxylin and mounted with Immunomount.

2.5.2 Indirect immunofluorescence on paraffin embedded sections

Double immunofluorescence labeling of tissues was carried out as described (Mason*, et al.* 2000). Paraffin-embedded sections (6 µm) were dewaxed in xylene and rehydrated. After antigen retrieval as described above (**2.5.1**), the slides were blocked with 10 % normal goat serum for 10 min at RT and subsequently incubated overnight at 4 °C with a mixture of the appropriate antibodies. Afterwards a mixture of highly cross absorbed secondary antibody (dilution 1:500) coupled to the appropriate Alexa fluorochromes was applied for 1 h at RT. The slides were mounted in glycerol 50 % in PBS. Between each step the slides were washed twice in PBS for 5 min.

For triple staining of GBP-1, CD31, and Ki67 slides were first stained for GBP-1/CD31 as described above and then incubated with 10% normal mouse serum at RT for 40 min. Subsequently, the slides were incubated with goat Fab anti-mouse fragments (1:20) at RT for 40 min. The slides were then incubated with the anti-Ki67 antibody (1:20). After washing the slides were incubated with a highly cross-absorbed goat anti-mouse antibody (1:500) coupled to the AlexaFluor350 fluorochrome. Between each step the slides were washed twice in PBS for 5 min. The slides were mounted in glycerol 50 % in PBS.

2.5.3 Immunocitochemistry

Cells were plated in 8-chamber glass culture slides and stimulated as described. After stimulation the cells were fixed in ethanol (20 min, 4 °C), methanol (4 min, -20 °C) or paraformaldehyd (PFA) (4 % ore 2 %, 20 min, RT). PFA fixed cells were permeabilized with 0.1 % Triton-X100 at RT (2 min). Slides were than stained using the Vectastain ABC kit as described in **2.5.1** for paraffin-embedded tissues without the dewaxing and the antigen retrieval steps.

2.5.4 Indirect immunofluorescence on fixed cells

Cells were plated in glass 8-chamber culture slides and treated as described in **2.5.3**. After fixation and permeabilization for PFA-fixed cells, unspecific binding sites were blocked with goat normal serum 10 %. Slides were then incubated with the primary antibody at the suitable dilutions ON at 4 °C. Afterwards, the slides were incubated with the appropriate secondary antibody coupled to AlexaFluor fluorochromes. Nuclei were visualised with DAPI at a final concentration of 1 µg/ml. Between each step the slides were washed twice in PBS for 5 min. Slides were mounted in glycerol 50 % in PBS. Stainings were observed using a LSM. Serial pictures taken along the z-axis where taken using the LSM5 software.

2.6 Enzyme-linked immunoassay (ELISA)

An ELISA for the detection of soluble GBP-1 was developed (Harlow 1999). The following buffers were used: PBS containing 0.1 % Tween 20 (PBS-T) and PBS containing 0.1 % Tween 20 and 2 % BSA (PBS-TB). Ninety-six-well immuno-plates were coated with 100 µl/well of anti-GBP-1 hybridoma supernatant (clone 1B1) diluted 1:5 in PBS for 16 h at 4 °C. Plates were rinsed with PBS-T and blocked with PBS-TB for at least 30 min at RT.

PBS-TB was aspirated and wells were incubated with 100 µl of samples in duplicates or triplicates for 2 h at RT. Recombinant purified GBP-1-His protein diluted in cell culture medium containing 5 % FBS was used as a standard; as a control for specificity BSA was used. Cell culture supernatants were used undiluted. Plasma and serum samples were diluted 1:2 or 1:8 in PBS in order to reduce the background signal.

Wells were then rinsed four times with PBS-T. Subsequently, 100 µl of rabbit polyclonal antibodies against GBP-1 (1:500 in PBS-TB) were added for 2 h at RT. After the wells were rinsed four times with PBS-T, 100 µl of alkaline phosphatase-conjugated anti rabbit antibody (1:500 in PBS-TB) were added for 1 h at RT. The wells were rinsed four times with PBS-T and incubated with 100 µl of p-nitrophenyl phosphate (PNPP). The absorbance was measured at 405 nm using a microplate reader. The concentration was calculated from the standard curve obtained from recombinant purified GBP-1-His protein. The concentration was calculated as a mean (±SD) of the value of three samples. The intra-assay variability was between 2.3 % and 6 %.

2.8 Statistical analysis

Statistical tests were performed using SPSS 8.0 for Windows. The Kolkogorov-Smirnov test was used to test the normal distribution of the data, a value of $p < 0.05$ was regarded as

significant. For normally distributed data differences were tested by a independent-sample T-test. A value of $p < 0.05$ was regarded as significant. Correlation was measured by Pearson correlation coefficient. A value of $p < 0.05$ was regarded as significant. If data were not normally distributed, differences between two independent samples were tested by Mann-Whitney test (2-tailed, if not other specified). A value of $p < 0.05$ was regarded as significant. Correlation was measured by Spearman´s rank correlation test or be Wilcoxon test. A value of $p < 0.05$ was regarded as significant.

RESULTS

1 Generation of mono- and polyclonal antibodies against GBP-1

In order to investigate the expression of GBP-1 at the protein level both *in vitro* and *in vivo*, specific antibodies against GBP-1 were produced.

1.1 Expression cloning and purification of recombinant GBP-1 proteins

Recombinant GBP-1 was purified and used as an immunogen in order to raise rat mono- and rabbit polyclonal antibodies. The full length cDNA sequence encoding GBP-1 was cloned into prokaryotic expression vectors in frame with a 6 x histidine (His) tag at the C-terminal (GBP-1-His) or at the N-terminal end (His-GBP-1).

In order to obtain a maximal yield of the recombinant proteins, the optimal conditions to induce the expression of soluble proteins in *E. coli* were investigated as described below.

E. coli cultures containing the prokaryotic expression plasmid encoding for GBP-1-His were stimulated with IPTG (100 μM) in order to induce expression of recombinant GBP-1-His protein. *E. coli* cultures were incubated at 37 °C or 25 °C and growth curves were determined. Induction of protein expression with IPTG reduced culture growth (data not shown). This indicated that overexpression of recombinant protein has a toxic effect on the cultures. This effect was more pronounced at 25 °C.

Subsequently, the soluble and insoluble protein fractions were extracted from IPTG-treated (100 μM, induction times indicated in Figure 9) *E. coli* cultures cultivated at 37 °C (Figure 9). The two fractions were analyzed by SDS-PAGE followed by Coomassie staining (Figure 9). The highest level of soluble recombinant GBP-1-His was observed at 4 h and 5 h after addition of IPTG (Figure 9). 5 h after addition of IPTG only the amount of insoluble GBP-1-His in the pellet was increased as compared to 4 h after addition of IPTG (Figure 9; compare P and S fractions at 5 h with the P and S fractions at 4 h). Therefore, for large scale induction of soluble recombinant GBP-1-His an induction time of 4 h was chosen.

After large scale induction of cultures containing the prokaryotic expression plasmid encoding for GBP-1-His, the respective protein was purified through a NiNTA sepharose column using fast performance liquid chromatography (FPLC) (Hochuli*, et al.* 1987). GBP-1-His eluted from the column in a single peak as shown by the chromatogram of the FPLC (Figure 10A).

Analysis of the elution fractions by SDS-PAGE and Coomassie staining showed that GBP-His was purified nearly to homogeneity (Figure 10B).

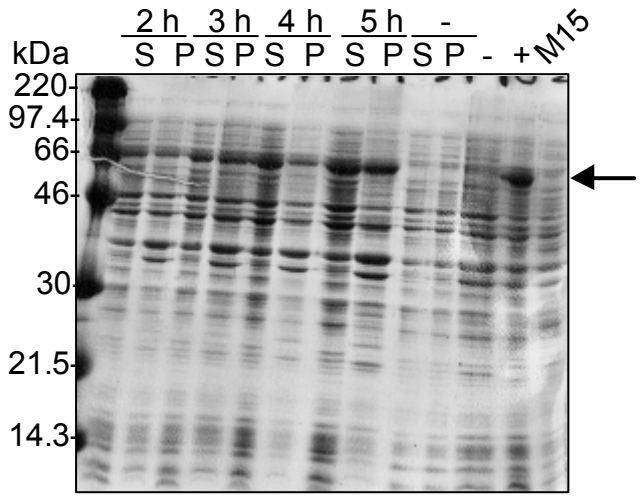


Figure 10: Determination of the optimal conditions for the induction and purification of recombinant GBP-1-His protein. *E. coli cultures were incubated at 37°C. GBP-1-His expression was induced using 100 µM IPTG for the indicated times. Non induced cultures (-) were used as a control. The soluble (S) and insoluble (P) fraction of proteins in IPTG induced (+) or non induced (-) cultures at were analyzed at each time point by SDS-PAGE and Coomassie staining. The following lysates were loaded as controls: (-), crude lysates of non induced cultures; (+), crude lysates of cultures induced with IPTG (100 µM) for 4 h; (M15), crude lysates of cultures without the expression vector. GBP-1-His is indicated by an arrow.*

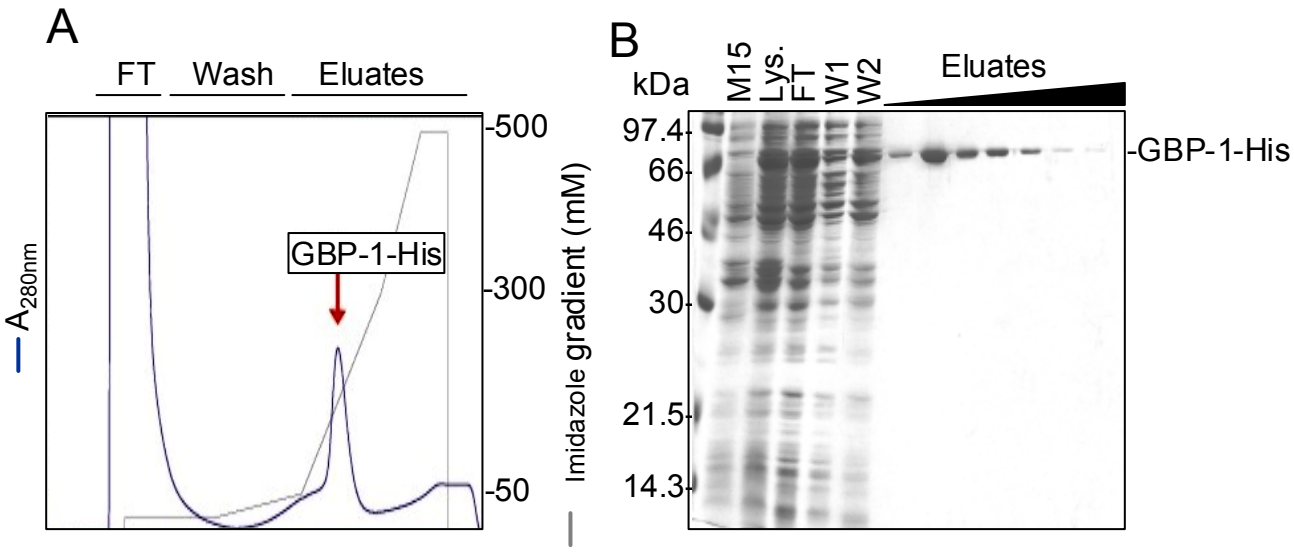


Figure 10: Analysis of the purification of recombinant GBP-1-His. *(**A**) Chromatogram of the elution fractions of GBP-1-His proteins after FPLC and NiNTA sepharose chromatography. An imidazole gradient was used for elution of recombinant GBP-1-His (gray line). The protein content was determined by measuring the absorbance of the flow through (FT), the wash and the eluted fractions at 280 nm (A_{280}) (blue line). (**B**) The proteins in the collected fractions were separated on SDS-PAGE and stained by Coomassie blue. M15 = lysates from M15 E. coli strain without the expression vector. Lys. = crude lysate of E. coli cultures containing the GBP-1-His expression vector after IPTG induction. FT = flow through. W1, W2 = wash fractions. Eluates = different fractions of the GBP-1-His elution peak represented in (**A**).*

In order to screen the specificity of the antibodies with a recombinant GBP-1 protein different from the one used for immunization, recombinant His-GBP-1 was purified (His tag at the N-terminus of GBP-1). For expression of soluble His-GBP-1 in *E. coli,* cultures were induced with 100 µM IPTG for 4 h at 37 °C. His-GBP-1 was purified through a NiNTA sepharose column using FPLC. His-GBP-1 eluted from the column in a single peak as shown by the chromatogram of the FPLC (Figure 11A). The elution fractions analyzed by Coomassie staining after separation on SDS-PAGE showed that His-GBP-1 was purified nearly to homogeneity (Figure 11B, eluates).

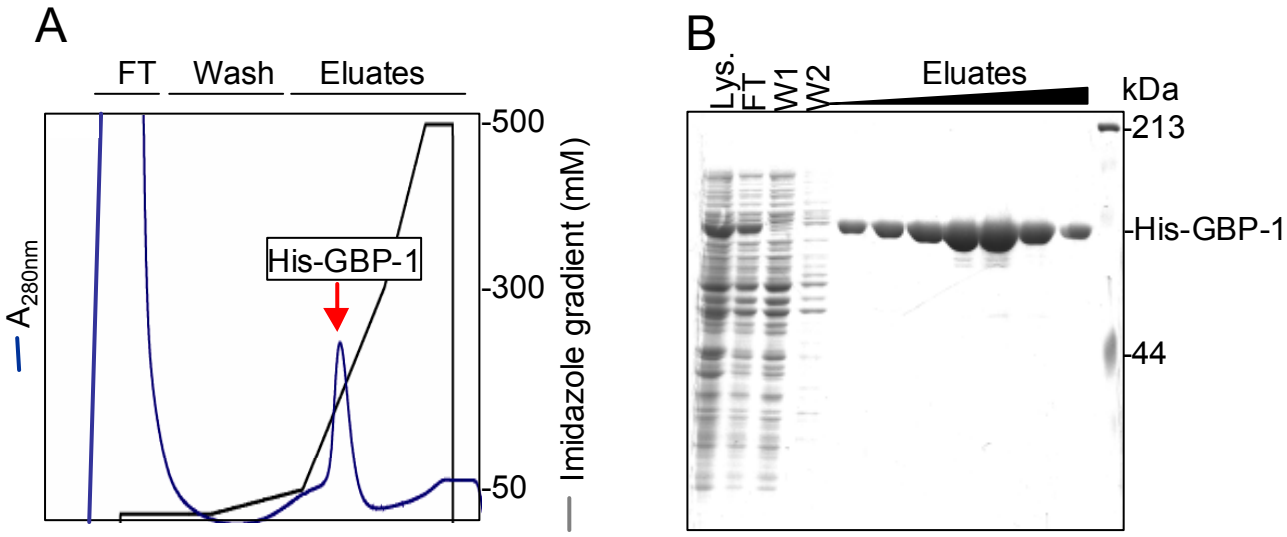


Figure 11: Analysis of the purification of recombinant His-GBP-1. *(**A**) Chromatogram of the elution fractions of recombinant His-GBP-1 after FPLC and NiNTA sepharose chromatography. An imidazole gradient was used for elution of His- GBP-1 (gray line). The protein content was determined by measuring the absorbance of the flow through (FT), the wash and the eluted fractions at 280 nm (A_{280}) (blue line). (**B**) The proteins in the collected fractions were separated on SDS-PAGE and stained by Coomassie blue. M15 = lysates from M15 E. coli strain without the expression vector. Lys. = crude lysate of E. coli cultures containing the His-GBP-1 expression vector after IPTG induction. FT = flow through. W1, W2 = wash fractions. Eluates = different fractions of His-GBP-1 elution peak represented in (**A**).*

In order to obtain proteins to test the specificity of the antibodies for the GBP-1 isoform, a recombinant His-GBP-2 protein was purified. Purification was performed under conditions analogue to the one of GBP-1-His (data not shown).

In order to get a control protein to exclude the possibility that antibodies recognized the His-tag of the recombinant GBPs, His-eGFP was purified. Expression of eGFP was induced in *E. coli* cultures containing the prokaryotic expression plasmid encoding for His-eGFP with IPTG (200 µM). *E. coli* cultures were incubated at 30 °C (Figure 12A) or 37 °C (Figure 12B) for the times indicated in Figure 12. Induction of protein expression with IPTG (200 µM)

reduced culture growth indicating that the expression of the recombinant protein has a toxic effect on the cultures compared to non-induced cultures (-), this effect was more pronounced at 30 °C (data not shown). In order to determine the optimal conditions to obtain a maximal yield of soluble recombinant His-eGFP, the soluble and insoluble protein fractions were extracted from IPTG-treated (200 μM) *E. coli* cultures. These cultures were cultivated at 37 °C (Figure 12A) or 30 °C (Figure 12B). The two fractions were analyzed by SDS-PAGE followed by Coomassie staining (Figure 12A and 12B).

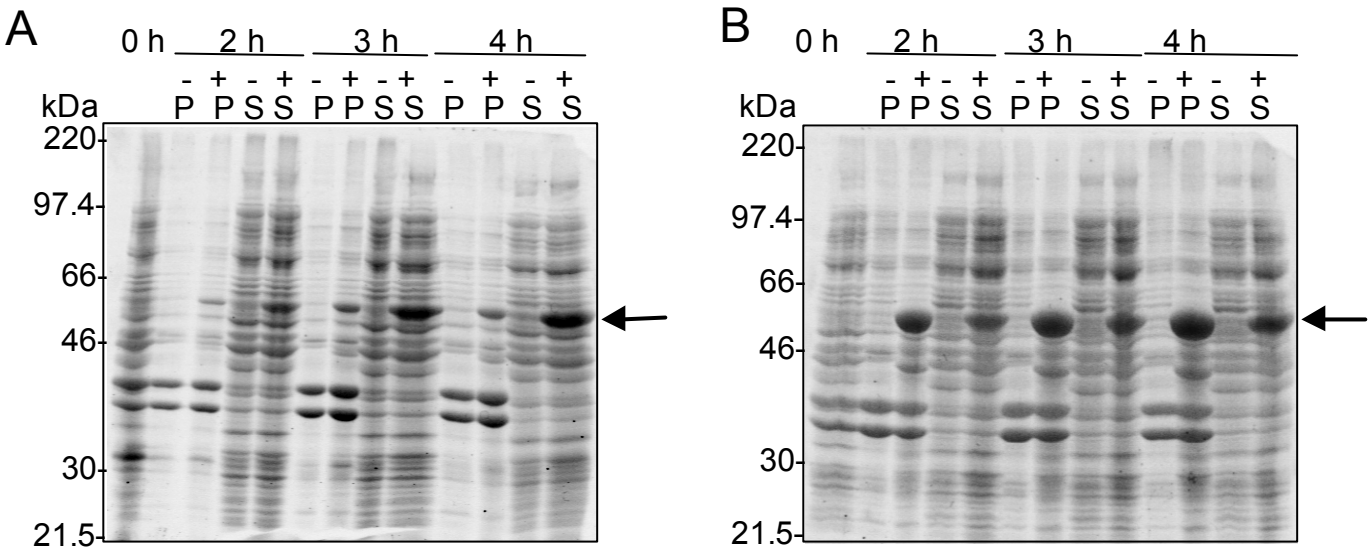


Figure 12: Determination of the best induction conditions for the purification of recombinant His-eGFP protein. *E. coli cultures were incubated either at 30 °C (**A**) or at 37 °C (**B**). His-eGFP expression was induced using IPTG (200 μM, +) for the indicated times. Non induced cultures (-) were used as a control. (**B** and **C**): The soluble (S) and insoluble (P) fraction of these cultures were analyzed at each time point by SDS-PAGE and Coomassie staining. Crude lysates of non induced cultures were loaded as a control (0). His-eGFP is indicated by an arrow*

The highest expression of soluble recombinant His-eGFP was observed at 3 h and 4 h after addition of IPTG (Figure 12B). Incubation of the cultures at 37 °C led to a higher expression of soluble recombinant His-eGFP (Figure 12B) as compared to incubation of the same cultures at 30 °C (Figure 12A). His-eGFP was purified near to homogeneity by FPLC using a NiNTA sepharose columns (data not shown).

1.2 Production of anti-GBP-antibodies

Monoclonal anti-GBP-1 antibodies (MAbs) were generated in collaboration with Dr. Elisabeth Kremmer (GSF, Munich) by immunization of LOU/C rats with purified

recombinant GBP-1 with a His tag in its C-terminal end (GBP-1-His, 50 µg). Four hybridoma clones were established. Three clones had an isotype IgG_1 (MAb 1B1, 6D9 and 5B9) and one clone had the isotype IgG_{2a} (MAb 6F12) (Table 2). Polyclonal anti-GBP-antibodies were generated by immunization of rabbits with purified GBP-1-His (500 µg), three antisera were obtained.

1.3 Characterization of anti-GBP-1 antibodies

In order to determine the reactivity of the different antibodies, purified His-GBP-1, His-GBP-2 and His-eGFP were separated by SDS-PAGE and analyzed by Western blot using MAb 1B1 (Figure 13, left panel), MAb 6F12 (Figure 13, right panel), MAb 6D9 and MAb 5B9 (data not shown). MAb 1B1 (Figure 13A, left panel) and MAb 5B9 (data not shown) reacted only with His-GBP-1, but not with His-GBP-2.

MAb 6F12 (Figure 13, right panel), MAb 6D9 and all polyclonal antibodies (data not shown) reacted with both His-GBP-1 and His-GBP-2. None of these antibodies recognized His-eGFP, indicating that the His-tag was not recognized by any of these antibodies (Figure 13A).

In order to investigate whether the antibodies were able to recognize GBP-1 in cell lysates, Western blot analysis of protein extracts obtained from HUVEC treated with IFN-γ (100 U/ml) for 16 h was performed. Only MAb 1B1, 6F12 and 6D9 recognized an IFN-γ-induced protein of 67 kDa in HUVEC (Figure 13B). No protein was recognized in the extracts of unstimulated HUVEC (Figure 13B, -). MAb 5B9 did not recognize any protein irrespective whether extracts of uninduced or IFN-γ-induced HUVEC were used (data not shown). The facts that MAb 1B1 (**i**) reacted specifically with the recombinant His-GBP-1, but not with His-GBP-2, (**ii**) recognized a protein of a molecular weight of 67 kDa that (**iii**) was selectively present in the cell extracts of IFN-γ stimulated HUVEC, indicated that the protein recognized in HUVEC was GBP-1. For further use of MAb 1B1 in Western blot analysis, its optimal dilution was titrated. MAb 1B1 worked best at a dilution 1:500. In this dilution it was able to recognize 10 ng of purified His-GBP-1.

In a next step the capability of the described antibodies to immunoprecipitate GBP-1 from cellular extracts was tested. HUVEC were treated with IFN-γ (100 U/ml) or BSA (-) for 24 h and immunoprecipitation of GBP-1 from the cell lysate was performed using polyclonal antibodies (Figure 14). The immunoprecipitate was analyzed by Western blot using MAb 1B1. GBP-1 could be immunoprecipitated from the cell lysate of stimulated (Figure 14, IFN-γ) but not from the cell lysate of unstimulated HUVEC (Figure 14, -).

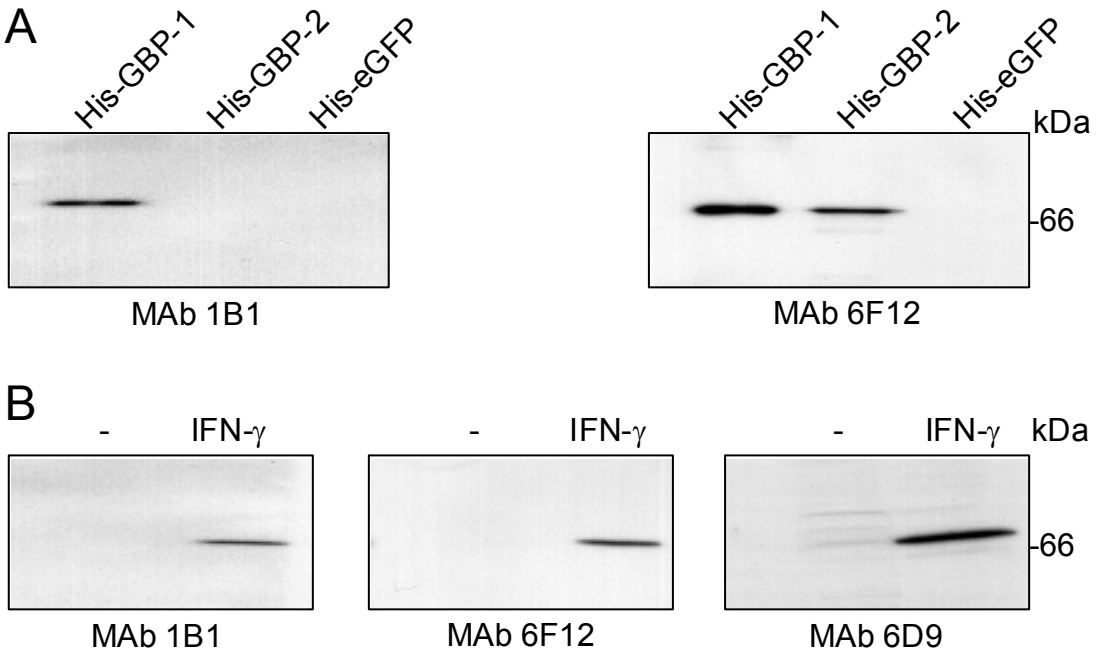


Figure 13: Western blot analyses with different monoclonal antibodies raised against GBP-1. *(**A**) Purified recombinant His-GBP-1, His-GBP-2 and His-eGFP (100 ng each) were separated by SDS-PAGE 10 %. Western blot analyses were performed using MAb 1B1 (left panel) and MAb 6F12 (right panel). All three the recombinant proteins had a His tag in their N-terminal end. (**B**) HUVEC were incubated with IFN-γ (100 U/ml) or BSA (-) for 16 h. 5 µg of total protein extract were loaded per line and separated by SDS-PAGE 10 %. Western blot analyses were performed using MAb 1B1, MAb 6F12 and MAb 6D9 as indicated in the figure.*

Immunoprecipitation was performed to completion as no GBP-1 was detected in the supernatant of the immunoprecipitate (Figure 14, Sup.). The respective pre-immunserum did not immunoprecipitate GBP-1 from the cell lysate of IFN-γ stimulated HUVEC (Figure 14, P.I.). In the immunoprecipitates the heavy chain of the antibodies used was detected (Figure 14, h.c.). None of the monoclonal antibodies was able to immunoprecipitate GBP-1 (data not shown). These results indicated that only the polyclonal antibodies worked for immunoprecipitation of GBP-1.

In order to test the ability of the antibodies to recognize GBP-1 at the single cell level, immunofluorescence studies were performed. HUVEC were treated with IFN-γ (100 U/ml) or BSA (-) for 24 h and fixed in methanol. GBP-1 was detected using indirect immunofluorescence (Figure 15), nuclei were counterstained with DAPI (Figure 15, blue). Unstimulated HUVEC did not show any GBP-1 staining (Figure 15, left panels). A clear signal was obtained with the monoclonal antibodies MAb 1B1 (Figure 15, red, right panel), MAb 6F12 (Figure 15, red, right panel), and 6D9 (data not shown) in IFN-γ-treated HUVEC only. No signals were obtained with MAb 5B9. With the polyclonal antibodies a high background staining was observed (data not shown). Similar results were obtained when cells were fixed in ethanol or paraformaldehyd (data not shown).

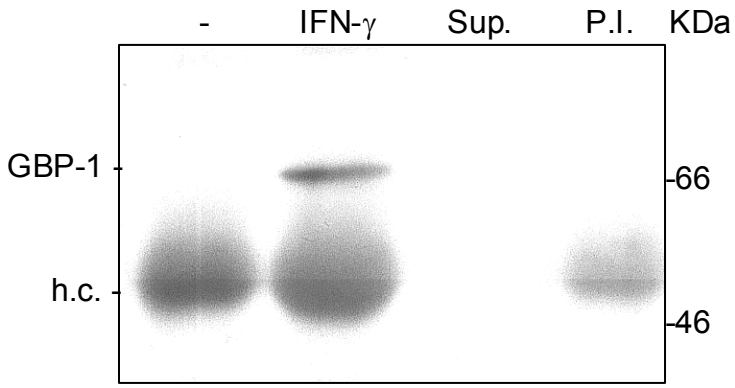


Figure 14: Western blot analysis of GBP-1 after immunoprecipitation of cellular GBP-1 using polyclonal rabbit anti-GBP antibodies. *Cellular GBP-1 from HUVEC incubated with IFN-γ (100 U/ml) or BSA (-) for 16 h was immunoprecipitated using a polyclonal anti GBP-1 antibody. Sup. = Supernatant after removal of the immunoprecipitate. P.I. = Immunoprecipitate of IFN-γ stimulated HUVEC using pre-immunserum. GBP-1 was detected by Western blot using MAb 1B1. The diffused bands are the heavy chains (h.c.) of the antibodies used for the immunoprecipitation.*

The results obtained on the specificity and the applicability of the different antibodies for the detection of GBP-1 with different methods are summarized in Table 2. The use of the different antibodies for the detection of GBP-1 in tissue sections is presented in detail in Paragraph **3**. MAb 1B1 was the only antibody that recognized specifically recombinant His-GBP-1, but not His-GBP-2. In addition, MAb 1B1 was the only GBP-1 specific antibody that recognized intracellular GBP-1 in IFN-γ-treated HUVEC, both in cell lysates and in fixed cells. Therefore, this anti-GBP-1 antibody was used for further analysis of GBP-1 expression in cultivated cells *in vitro* and tissues *in vivo*.

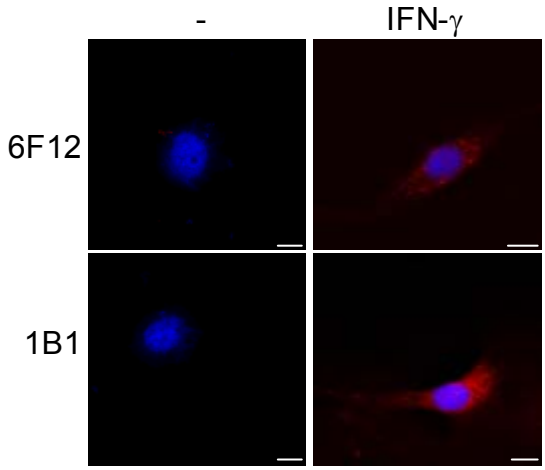


Figure 15: Detection of GBP-1 by indirect immunofluorescence in fixed HUVEC. *HUVEC were incubated with IFN-γ (100 U/ml) or with BSA (-) for 24 h and fixed with methanol. Indirect immunofluorescence analysis using the indicated MAbs was performed. GBP-1 is stained in red. Nuclei were counterstained with DAPI (blue). Scale bar = 10 μm.*

Table 2: Anti GBP-1 antibodies and their application in different methods.

	Antigen	His-GBP-1	His-GBP-2	GBP-1 in cells			
Reactivity	Application	WB			IP	IF	IHC
Rat mono-clonal antibodies	6F12 (IgG_{2a})	+	+	+	-	+	+
	6D9 (IgG_1)	+	+	+	-	+	+
	1B1 (IgG_1)	+	-	+	-	+	+
	5B9 (IgG_1)	+	-	-	-	-	-
Rabbit polyclonal antibodies	Rabbit 1	+	+	+	+	*n.d.*	*n.d.*
	Rabbit 2	+	+	+	+	*n.d*	*n.d*
	Rabbit 3	+	+	+	+	*n.d*	*n.d*

WB = Western blot, IP = immunoprecipitation, IF= immunofluorescence, IHC = immunohistochemistry, Cell lysate. = GBP recognized in cell extracts, n.d. = non determined, + = positive reaction, - = negative reaction. The isotype of the respective antibodies is given in branches.

2 Characterization of GBP-1 expression in inflammatory cytokine-activated endothelial cells *in vitro*

2.1.1 Effects of inflammatory cytokines on GBP-1 expression

Previous work in this laboratory had shown that GBP-1 mRNA is induced by inflammatory cytokines (IC) in EC. Here the MAb 1B1 was used to investigate the expression of GBP-1 protein under the same conditions.

HUVEC were stimulated with IFN-γ (100 U/ml), IFN-α (100 U/ml), IL-1ß (20 U/ml) and TNF-α (300 U/ml) for 24 h. Afterwards, GBP-1 expression was analyzed by Western blot (Figure 16). In all IC stimulated HUVEC GBP-1 protein expression was higher as compared to non stimulated control cells (Figure 16). IFN-γ had the strongest effect, whereas IFN-α, IL-1ß and TNF-α had a less pronounced effect on GBP-1 expression (Figure 16). The same results as observed in HUVEC were obtained in dMVEC (data not shown). Also lower concentrations of these cytokines induced GBP-1 expression in HUVEC: IFN-γ (1 U/ml), IL-1ß (2 U/ml) and TNF-α (30 U/ml) for 16 h (Figure 17). This indicated that GBP-1 expression in EC may be induced already by physiological IC concentrations (Breuer-McHam*, et al.* 1998).

In order to investigate the time course of GBP-1 expression, HUVEC were stimulated with IFN-γ (100 U/ml) and cell extracts were harvested at different times as indicated in Figure 18. Western blot analysis of GBP-1 expression indicated that GBP-1 was detectable 4 h after stimulation and increased continuously for 24 h after stimulation (Figure 18).

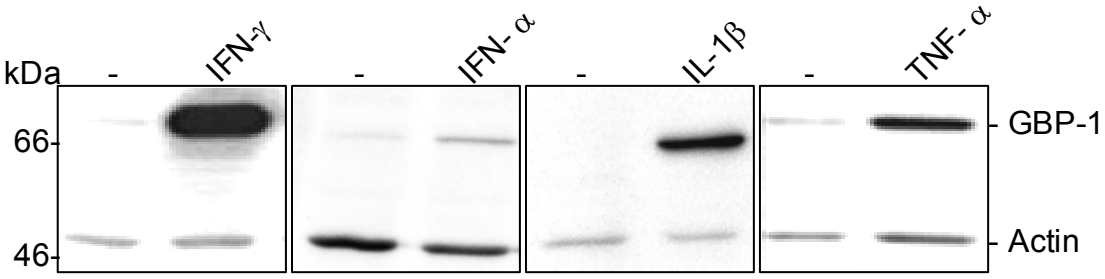


Figure 16: Western blot analysis of GBP-1 expression in HUVEC in the presence of IC. *HUVEC were incubated with IFN-γ (100 U/ml), IFN-α (100 U/ml), IL-1β (20 U/ml) and TNF-α (300 U/ml) or BSA (-) for 24 h. GBP-1 expression was analyzed by Western blot using MAb 1B1. Actin staining shows that similar amounts of protein extracts were loaded.*

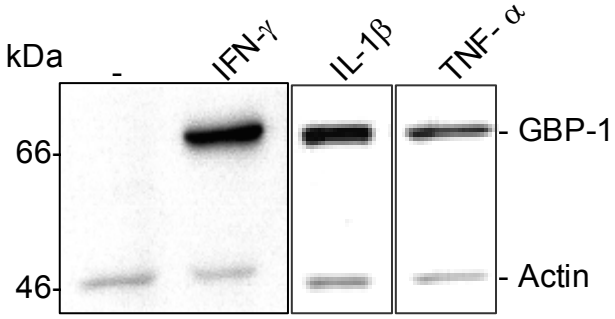
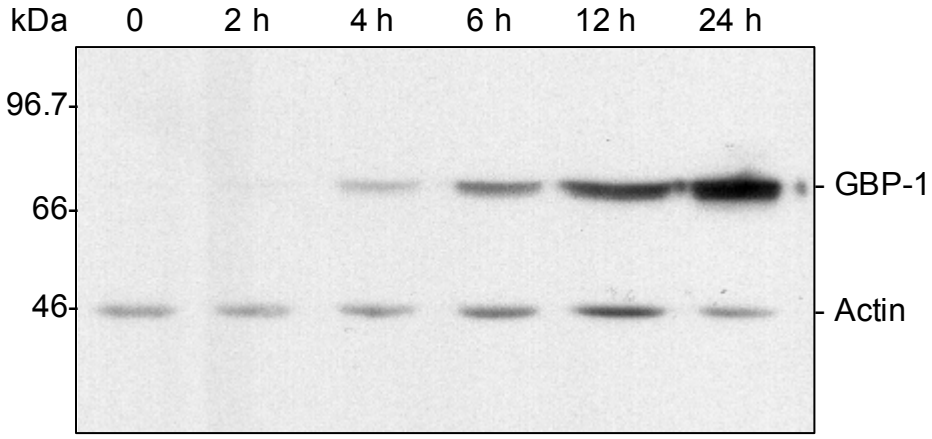


Figure 17: Western blot analysis of GBP-1 expression in HUVEC in the presence of low IC concentrations. *HUVEC were incubated with IFN-γ (1 U/ml), IL-1β (2 U/ml), TNF-α (30 U/ml) or BSA (-) for 16 h. GBP-1 expression was analyzed by Western blot using MAb 1B1. Actin staining shows that comparable amounts of protein extracts were loaded. From (Guenzi et al. 2001).*



Figures 18: Western blot analysis of the kinetic of IFN-γ-induced GBP-1 expression in HUVEC. *HUVEC were incubated with IFN-γ (100 U/ml) for the indicated times and GBP-1 expression was analyzed by Western blot using MAb 1B1. Actin staining shows that comparable amounts of protein extracts were loaded.*

IL-1ß has been shown to stimulate IFN-γ expression in natural killer cells (Cooper, *et al.* 2001). Therefore, the possibility that IL-1ß-induced GBP-1 protein expression may be mediated *via* IFN-γ was investigated. The amount of IFN-γ in the cell culture supernatants of IL-1ß-treated HUVEC was measured using a commercial ELISA (Figure 19). A commercial IFN-γ standard

diluted in endothelial cell medium (EBM-2) was used for quantification (Figure 19, gray bars). As a positive control for the ability of the ELISA to detect IFN-γ in cell culture supernatants, the cell culture supernatant of HuT 78 cells stimulated with IL-12 and IL-18 (10 ng each) for 5 h was used. Stimulation of HuT 78 cells with IL-12 and IL-18 has been shown to induce secretion of IFN-γ (Tripp*, et al.* 1993; Walker*, et al.* 1999). IFN-γ was detected in the cell culture supernatants of IL-12 + IL-18 stimulated HuT 78 cells as expected (Figure 19, black bar). The amount of IFN-γ in the cell culture supernatants of HUVEC stimulated with IL-1ß for 24 h was below of the sensitivity of the ELISA (8 pg/ml corresponding to an OD of 0.06) (Figure 19, red line). The same results were obtained when HUVEC were stimulated with the same concentrations of IL-1ß for 5 h (data not shown).

Altogether these data indicated that GBP-1 expression in HUVEC is induced directly by IL-1ß and is not due to the induction of IFN-γ expression in these cells.

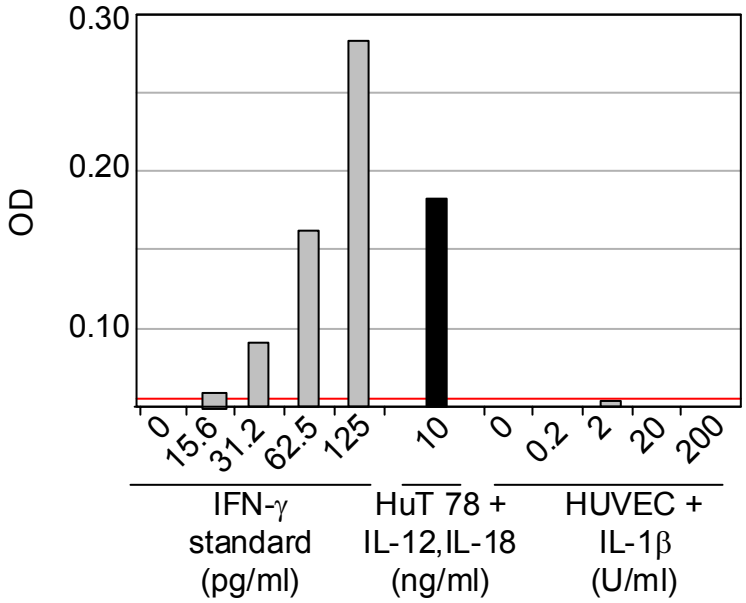


Figure 19: Detection of IFN-γ in cell culture supernatants by ELISA. *A commercial IFN-γ ELISA was used to measure IFN-γ concentrations in the cell culture supernatants of IL-1ß-treated HUVEC. IFN-γ standards were diluted in endothelial cell medium (EBM-2, gray bars). As a control HuT 78 cells were incubated with IL-12 + IL-18 at the indicated concentrations for 5 h (black bar). HUVEC were incubated with the indicated IL-1β concentrations for 24 h. OD = corrected optical density (absorbance at 450 nm - absorbance at 570 nm). The sensitivity of the ELISA was 8 pg/ml corresponding to an OD of 0.06 (red line).*

In a next step, the effects of repeated IC stimulation on GBP-1 expression in EC were investigated. HUVEC were incubated in low medium for 16 h and then simulated with IL-1ß (20 U/ml). After 24 h the medium was changed and fresh low medium was added either alone or supplemented with IL-1ß for further 32 h. GBP-1 expression was analyzed by Western blot.

GBP-1 expression was stable up to 56 h. Notably, in HUVEC that expressed GBP-1 after a first IL-1ß stimulation GBP-1 expression could be further induced with a second IL-1ß stimulation (data not shown). The same results as observed in HUVEC were obtained in dMVEC in a similar experiment. Altogether these results indicated GBP-1 expression is stable for days and that repeated IL-1ß treatment of EC is able to stabilize GBP-1 expression.

2.1.2 Effects of angiogenic growth factors on inflammatory cytokine-induced GBP-1 expression

Recently it has been shown from this laboratory that IC-induced GBP-1 mRNA is downregulated when angiogenic growth factors (AGF) were added simultaneously with IC to EC.

In order to investigate the effect of AGF on IC-induced GBP-1 expression at the protein level, GBP-1 expression was analyzed by Western blot in cell lysates of HUVEC and dMVEC, incubated with IFN-γ (100 U/ml), IL-1β (200 U/ml) and TNF-α (300 U/ml) alone or in the presence of AGF (VEGF and bFGF, 10 ng/ml each) for 24 h (Figure 20A and 20B, upper panels).

GBP-1 expression was highly expressed in the presence of IC (Figure 20A and 20B). Notably, in presence of AGF the IC-induced GBP-1 expression was significantly lower as compared to cells treated with IC alone both in HUVEC (Figure 20A) and in dMVEC (Figure 20B). These results confirmed at the protein level that IC-induced GBP-1 expression is inhibited by AGF when the factors are present simultaneously.

In a next step it was investigated wether IC can induce GBP-1 expression in EC that have been pre-incubated with AGF. HUVEC were incubated in low medium alone or with AGF (VEGF and bFGF, 10 ng/ml each) for 16 h and then incubated with IFN-γ (100 U/ml), IL-1ß (20 U/ml) or TNF-α (300 U/ml) for 24 h. Subsequently, GBP-1 expression in these cells was analyzed by Western blot (Figure 21). No difference was observed in the induction of GBP-1 expression by IC, irrespective whether the cells have been pre-incubated with AGF or not. The same results as observed in HUVEC were obtained in dMVEC (data not shown).

Altogether these results confirmed at the protein level that AGF applied simultaneously with IC inhibit IC-induced GBP-1 expression, whereas pre-incubation of EC with AGF for 16 h does not affect IC-induced GBP-1 expression, both in HUVEC and in dMVEC.

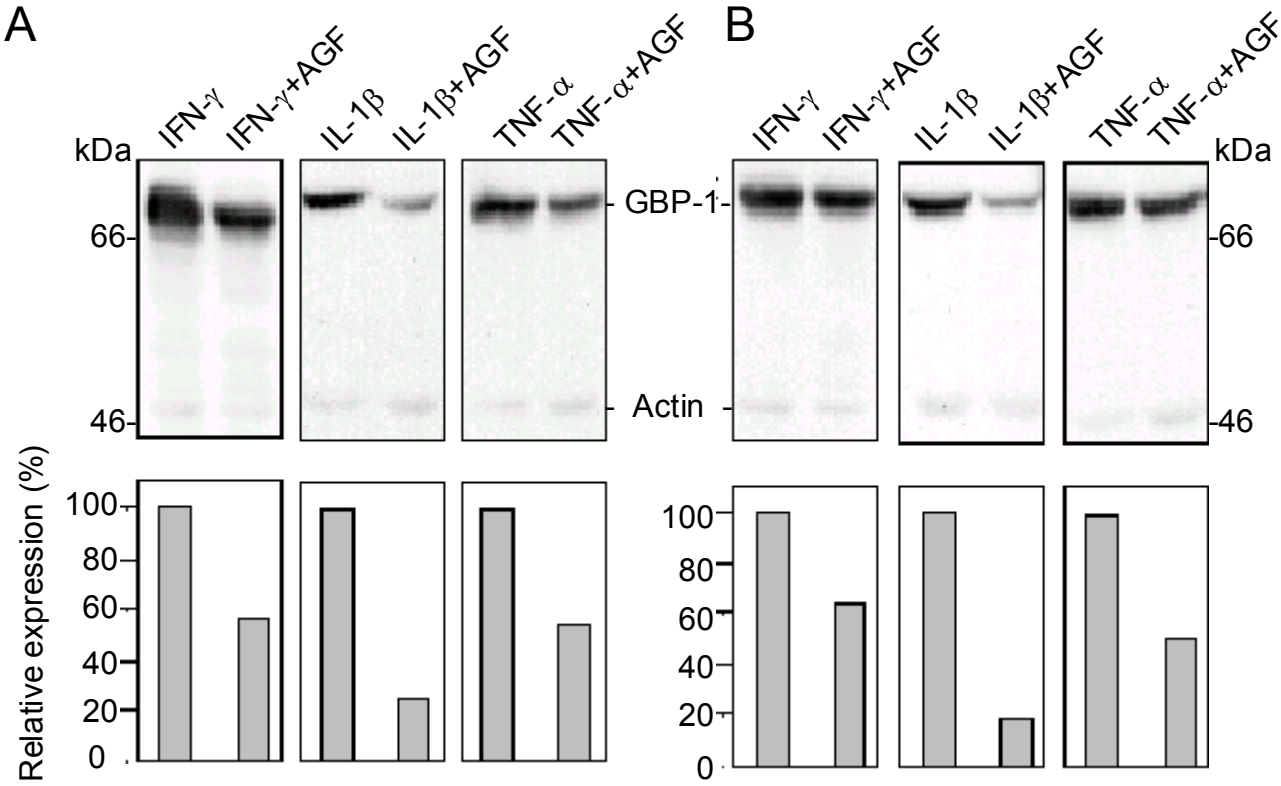


Figure 20: Western blot analysis of IC-induced GBP-1 expression in the simultaneous presence of AGF. *(**A**) HUVEC and (**B**) dMVEC were incubated with IFN-γ (100 U/ml), IL-1β (200 U/ml) and TNF-α (300 U/ml) alone or in the presence of AGF (VEGF and bFGF, 10 ng/ml each) for 24 h. GBP-1 expression was analyzed by Western blot using MAb 1B1 (upper panels). Corresponding signal intensities were densitometrically determined (lower panels, gray bars). From (Guenzi et al. 2001).*

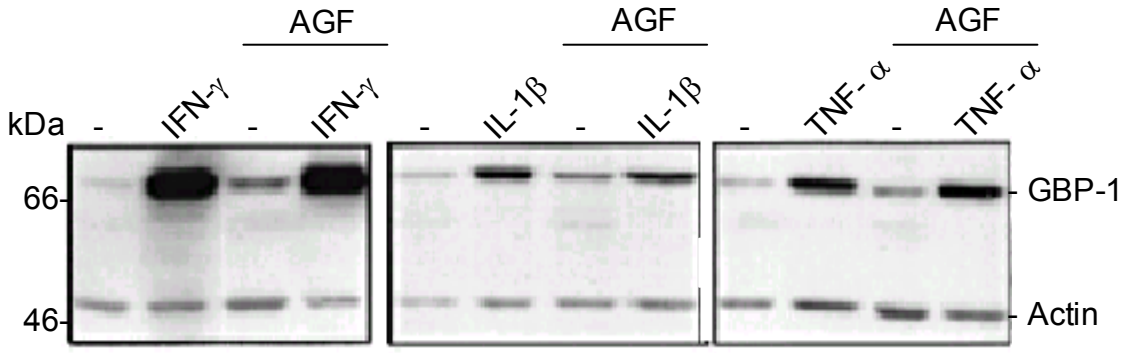


Figure 21: Western blot analysis of IC-induced GBP-1 expression in HUVEC after pre-incubation with AGF. *HUVEC were pre-incubated for 16 h in presence or absence of AGF (VEGF and bFGF, 10 ng/ml each) and then incubated with IFN-γ (100 U/ml), IL-1β (20 U/ml), TNF-α (300 U/ml) or BSA (-) for 24 h. GBP-1 expression was analyzed by Western blot using MAb 1B1. Actin staining shows that comparable amounts of protein extracts were loaded. From (Guenzi et al. 2001).*

2.1.3 Effects of different factors on GBP-1 expression

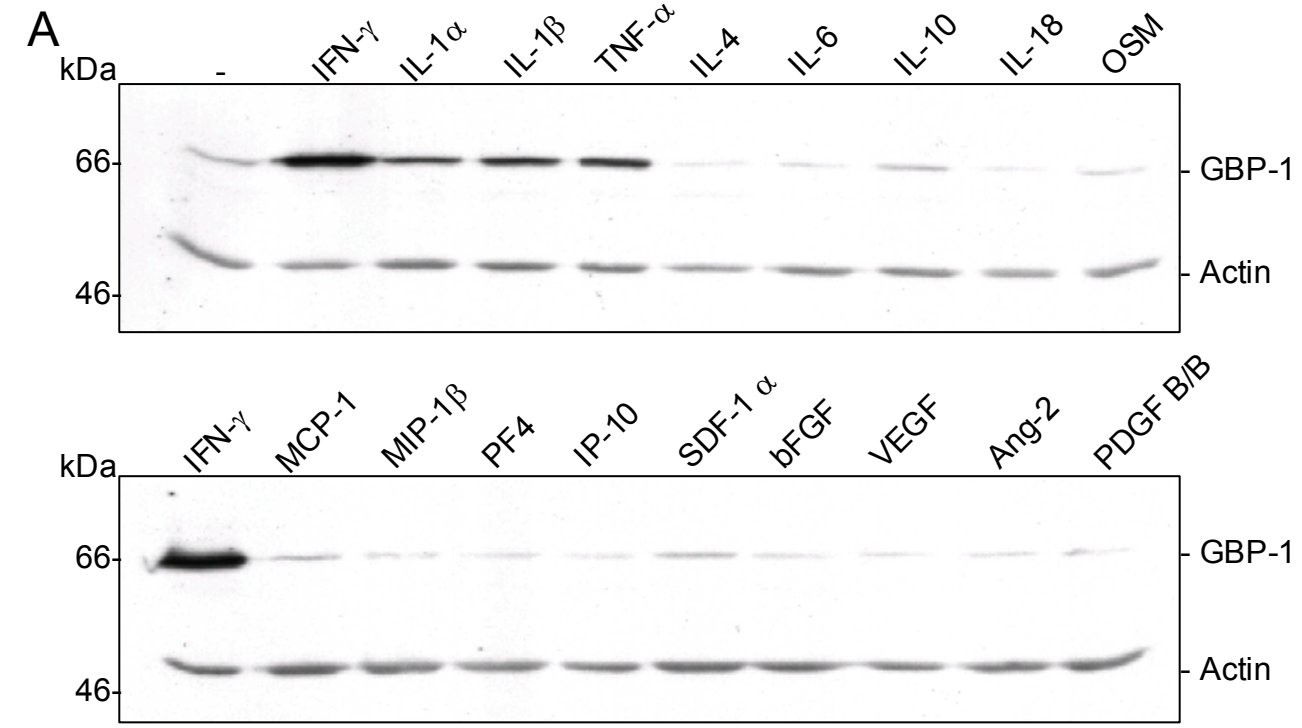
In order to investigate which other factors besides IC may induce GBP-1 expression in HUVEC the following experiments were performed. HUVEC were treated with IFN-γ as a positive control or with different factors including cytokines [IL-1α, IL-1β, TNF-α, IL-4, IL-6, IL-10, IL-18, oncostatin M (OSM)], C-C chemokines (MCP-1, MIP-1β), C-X-C chemokines (PF4, IP-10, SDF-1α) and growth factors (bFGF, VEGF, Ang-2, PDGF B/B) for 24 h.

In the concentrations used, each factor induced a clear biological response in HUVEC. VEGF and IL-18-induced chemotaxis of HUVEC (Figure 22B). For each chemokine subfamily one member was tested for its effect on EC. The C-C chemokine MCP-1 and the C-X-C chemokine SDF-1α-induced chemotaxis of HUVEC (Figure 22B). IL-4 and IL-1α up-regulated VCAM-1 expression in HUVEC (Figure 22C). HUVEC proliferation was activated by Ang-2, VEGF (data not shown), bFGF and PDGF B/B (Figure 22D). In contrast, HUVEC proliferation was inhibited by OSM (Figure 22D), as well as by IFN-γ, IL-1β, IL-6. IL-10 and TNF-α (data not shown), (Guenzi, et al. 2001).

All these findings were in agreement with previously published activities of these factors on HUVEC (Folkman, et al. 1987; Bevilacqua, et al. 1989; Ferrara, et al. 1989; Keck, et al. 1989; Cavender, et al. 1991; Swerlick, et al. 1992; Holzinger, et al. 1993; Cornali, et al. 1996; Haraldsen, et al. 1996; Takashima, et al. 1996; Romero, et al. 1997; Gentilini, et al. 1999; Salcedo, et al. 1999; Salcedo, et al. 2000; Guenzi, et al. 2001; Lee, et al. 2001; Park, et al. 2001).

Western blot analysis of GBP-1 expression in HUVEC under these different conditions revealed that GBP-1 expression was selectively induced by IC including IFN-γ, IL-1α, IL-1β, and TNF-α, but by none of the other factors (Figure 22A). Altogether these data indicated that GBP-1 characterizes IC-activated EC. Notably, all these IC, which induced GBP-1 expression in EC, have been shown to inhibit proliferation of these cells (Frater-Schroder*, et al.* 1987; Friesel*, et al.* 1987; Schweigerer*, et al.* 1987a; Cozzolino*, et al.* 1990; Ruszczak*, et al.* 1990; Guenzi*, et al.* 2001)

Figure 22: Effect of different factors on GBP-1 expression in HUVEC.
*(**A**) HUVEC were incubated with the indicated factors or with BSA (-) for 24 h. The following concentrations were used: IFN-γ (100 U/ml), IL-1α (5 ng/ml), IL-1β (200 U/ml), TNF-α (300 U/ml), IL-4 (10 U/ml), IL-6 (50 U/ml), IL-10 (50 ng/ml), IL-18 (100 ng/ml), OSM, (10 ng/ml), MCP-1 (50 ng/ml), MIP-1β (50 ng/ml), PF4 (25 ng/ml), IP-10 (50 ng/ml), SDF-1α (200 ng/ml), bFGF (10 ng/ml), VEGF (10 ng/ml), Ang-2 (800 ng/ml) and PDGF B/B (100 ng/ml) (Figure 22A). GBP-1 expression was analyzed by Western blot using MAb 1B1. Actin staining shows that comparable amounts of protein extracts were loaded. From (Lubeseder-Martellato et al. 2002). Biological activity of the concentrations of the different factors used in HUVEC is demonstrated in (**B - D**)*
*(**B**) HUVEC were incubated with SDF-1α (200 ng/ml) for 2 h or VEGF (10 ng/ml), IL-18 (100 ng/ml) and MCP-1 (50 ng/ml) for 4 h. As a control low medium (LM) was used. Briefly chemotaxis experiments were performed using Boyden chambers. Polycarbonate filters of 8 µm pore size were coated with 1.5% bovine skin gelatine. Low medium with or without the indicated factors was placed in the lower compartment of the chamber. 2 x 10^4 cells were added into the upper compartment. After incubation at 37 °C the filters were harvested. The cells were removed from the upper side and transmigrated cells at the lower side were fixed with methanol at –20 °C for 4 min and stained with Haematoxylin. The numbers of migrated cells was determined under the microscope. The results are expressed as the mean number (± SD) of migrated cells/5 microscopic fields (25 x magnification). Each experiment was performed in triplicate.*
*(**C**) HUVEC were incubated with IL-4 (10 U/ml), IL-1α (5 ng/ml) or BSA (-) for 24 h. VCAM-1 expression was analyzed by Western blot using a polyclonal rabbit antibody. Actin staining shows that comparable amounts of protein extracts were loaded.*
*(**D**) HUVEC were incubated with PDGF B/B (100 ng/ml), bFGF (10 ng/ml), OSM (10 ng/ml), or simultaneously with PDGF B/B and bFGF or OSM and bFGF at the same concentrations. LM = cells grown in low medium. Briefly, proliferation experiments were performed by seeding HUVEC (10^3 or 10^4 cells/ml) into 24-well plates. Then HUVEC were incubated for 16 h in low medium (t0). Subsequently cytokines and growth factors were added at the indicated concentrations. After three days cell numbers were determined. Each stimulation was carried out in triplicate. The results are expressed as the mean of the cell numbers (± SD).*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kDa
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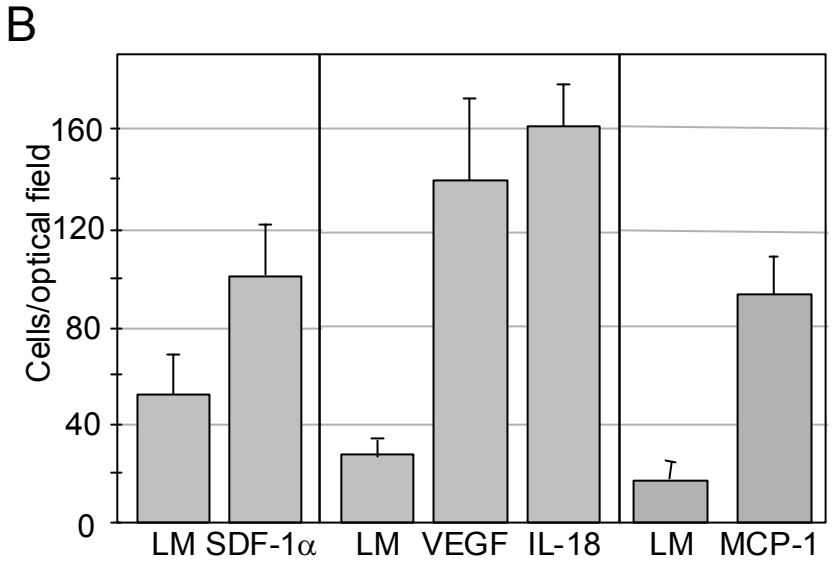
IFN-γ
IL-1α
IL-1β
TNF-α
IL-4
IL-6
IL-10
IL-18
OSM
66
46
GBP-1
Actin
kDa
IFN-γ
MCP-1
MIP-1β
PF4
IP-10
SDF-1 α
bFGF
VEGF
Ang-2
PDGF B/B
66
46
GBP-1
Actin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Cells/optical field
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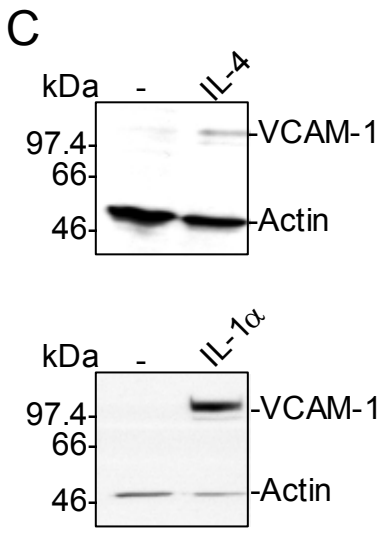
120
80
40
0
LM
SDF-1α
LM
VEGF
IL-18
LM
MCP-1


C
kDa
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IL-4
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VCAM-1
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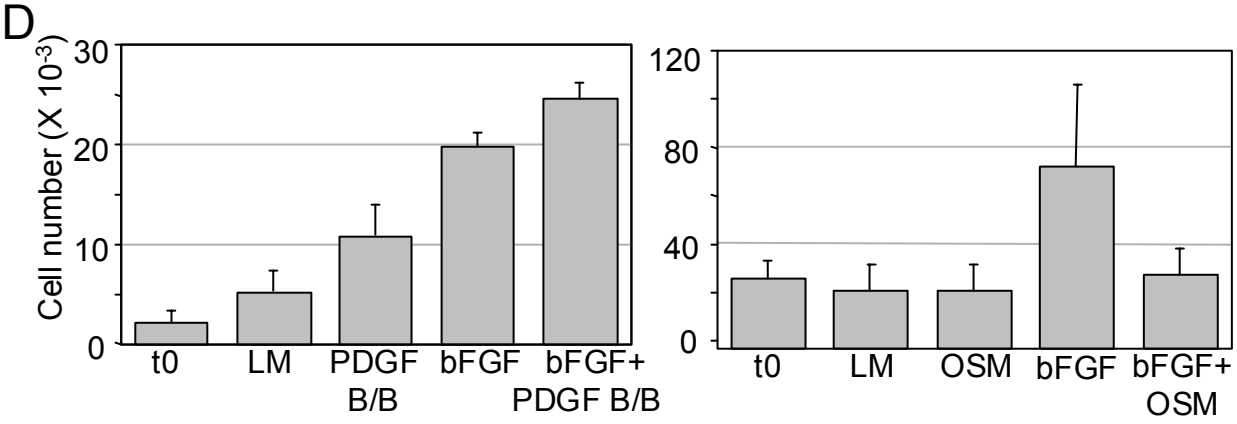

D
Cell number (X 10-3)
30
20
10
0
t0
LM
PDGF B/B
bFGF
bFGF+ PDGF B/B
120
80
40
0
t0
LM
OSM
bFGF
bFGF+ OSM

2.2 Studies of GBP-1 subcellular localization in HUVEC

In order to investigate whether GBP-1 subcellular localization was different in HUVEC incubated with different IC, immunocytochemical and immunofluorescence studies were performed. In a first approach GBP-1 localization was investigated by immunohistochemistry in HUVEC stimulated with IFN-γ (100 U/ml) for 16 h (Figure 23, IFN-γ). GBP-1 was selectively localized in the cytoplasm. No signal was observed in unstimulated cells (Figure 23, -). Identical results were observed when HUVEC were stimulated with IL-1β (20 U/ml) or TNF-α (300 U/ml) (data not shown).

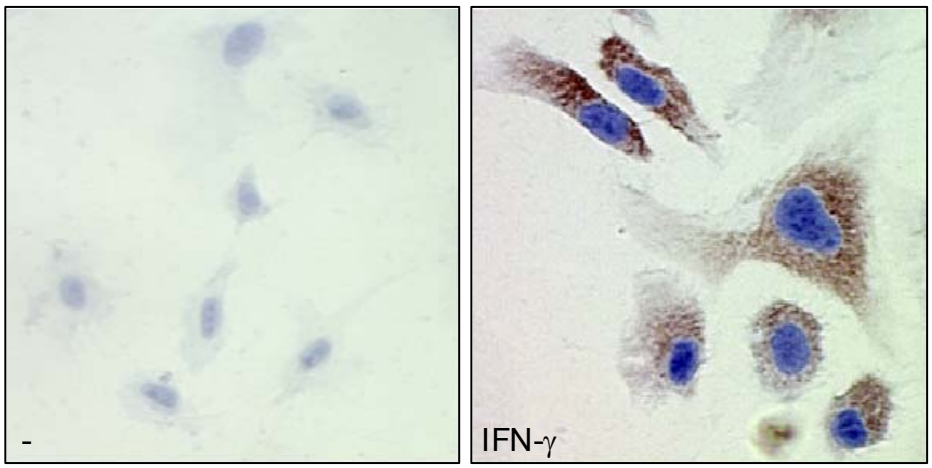


Figure 23: Immunocytochemical analysis of GBP-1 localization in HUVEC. *HUVEC were incubated with IFN-γ (100 U/ml) or with BSA (-) for 24 h and fixed with methanol. For detection of GBP-1 a standard immunoperoxidase staining procedure was used with MAb 1B1. Nuclei were counterstained with hematoxilin (blue), GBP-1 appears as a brown cytoplasmic staining. Magnification: x 630. From (Lubeseder-Martellato et al. 2002).*

In a next set of experiments, GBP-1 localization was investigated by indirect immunofluorescence in HUVEC stimulated with IFN-γ (100 U/ml), IL-1β (20 U/ml) or TNF--α (300 U/ml) for 24 h (Figure 24, see also Figure 15). GBP-1 localized exclusively in the cytoplasm of IC-stimulated HUVEC and was present in some granular structures (Figure 24B-24C, white arrows). Unstimulated cells showed no staining (Figure 24A).
These results indicated that GBP-1 subcellular localization in HUVEC is the same, irrespectively of the cytokine used for induction of GBP-1 expression.

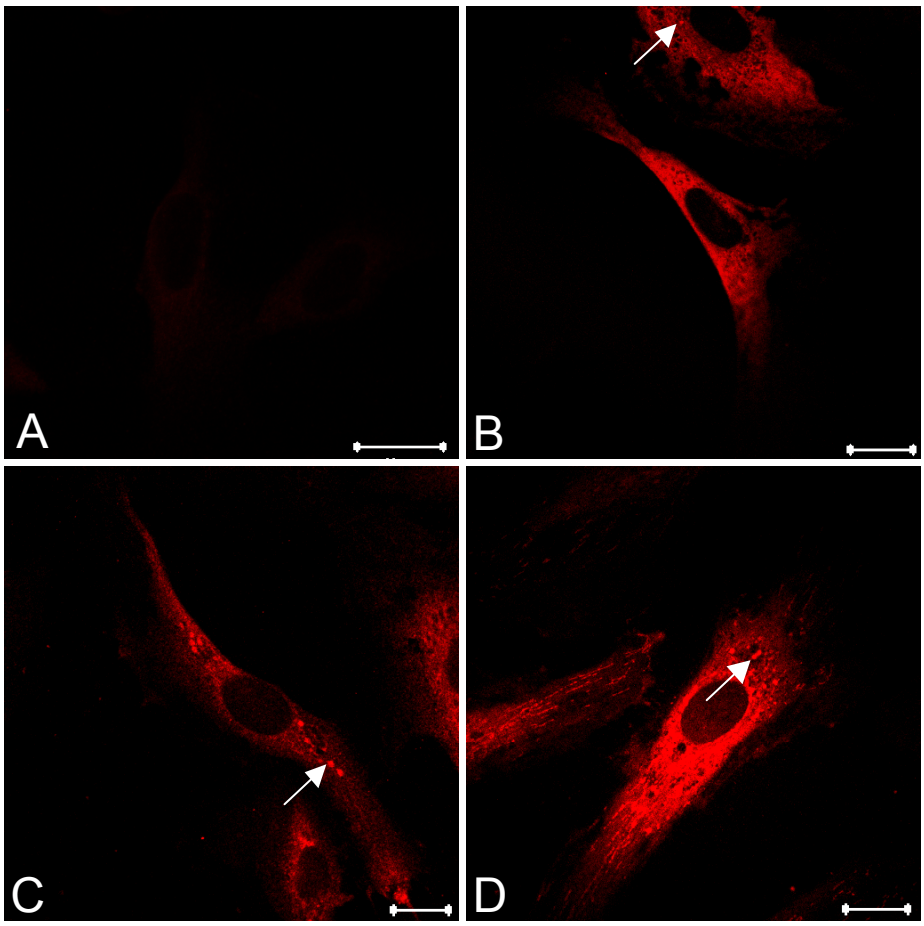


Figure 24: Immunofluorescence analysis of GBP-1 subcellular localization in HUVEC. *HUVEC were incubated with BSA (**A**), IFN-γ (100 U/ml) (**B**), IL-1β (20 U/ml) (**C**) or TNF-α (300 U/ml) (**D**) for 24 h and fixed with methanol. For detection of GBP-1 MAb 1B1 was used. Granular structures are indicated by white arrows. Scale bar = 20 μm.*

2.3 Colocalization studies of GBP-1 with markers for different organelles

In order to investigate whether the granular staining reaction of GBP-1 may be due to localization of GBP-1 in intracellular vesicles, double-immunofluorescence studies were performed for the detection of GBP-1 and different markers specific for defined organelles.

GBP-1 expression in HUVEC incubated with IFN-γ (100 U/ml) (Figure 25A and 25D) or IL-1β (20 U/ml) (Figure 25B and 25C) for 25 h was analyzed by indirect immunofluorescence (Figure 25, red). In these cells, GBP-1 was stained using MAb 1B1 (Figures 25A-D, red arrows). In addition, in these cells caveolae were stained using anti-caveolin-1 (Figure 25A, green) and anti-P-caveolin antibodies (Figure 25B, green). GBP-1 granular staining did not colocalize with caveolae (Figures 25A and 25B). In a next step, lysosomes were stained using cathepsin-D (Figure 25C; green). GBP-1 granular structures

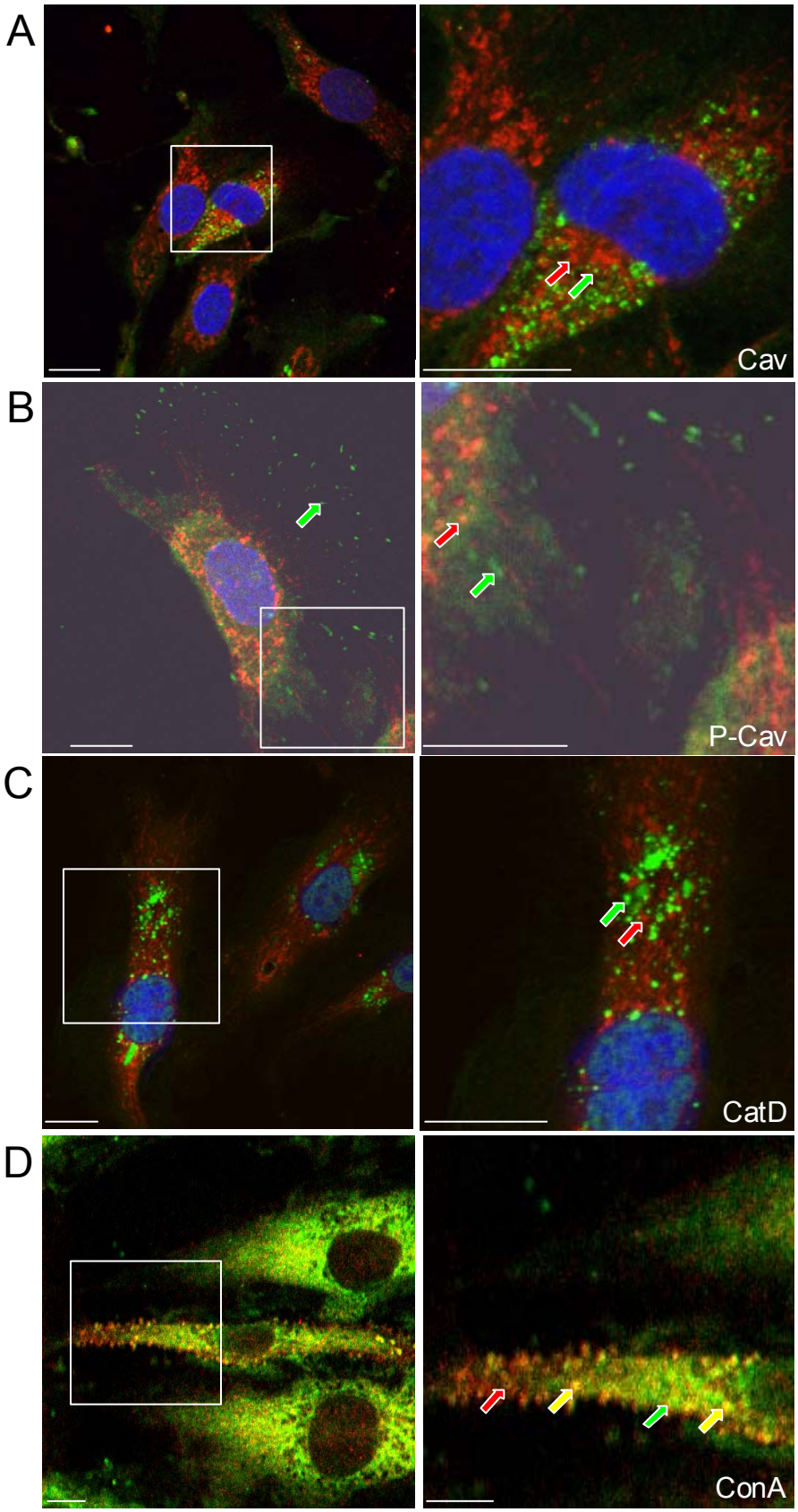


Figure 25: Double immunofluorescence stainings of IC-stimulated HUVEC. *HUVEC were incubated with IFN-γ (100 U/ml) (**A** and **D**) or IL-1β (20 U/ml) (**B** and **C**) for 24 h and then fixed with methanol. For detection of GBP-1 MAb 1B1 was used. Red arrows indicate GBP-1 granular structures. Cellular organelles distinct from GBP-1 aggregates are indicated by a green arrow. Caveolae were stained with anti-caveolin (Cav, **A**, green) and anti-phospho-caveolin antibodies (P-caveolin, **B**, green). (**B**) Phospho-caveolin is concentrated more at the periphery of the cell (green arrow). Lysosomes were stained with cathepsin D Cav, **B**). In (**A**, **B** and **C**) nuclei where counterstained with DAPI (blue). The endoplasmic reticulum was stained with ConA (**D**, green). For each staining a magnification of the white insert is shown in the right panels. In (D) yellow arrows indicate GBP-1 colocalization with ConA. Scale bar = 20 μm.*

were also distinct from lysosomes. In addition GBP-1 did not colocalize with stainings obtained with anti-Lamp-1 and anti-Rab7 antibodies for lysosomes and anti-TGN38

antibodies for the Golgi apparatus (data not shown). Finally the endoplasmic reticulum was stained using the lectin concanavalin A (ConA) (Figure 25D, green). ConA binds selectively to α-mannopyranosyl and α-glucopyranosyl residues. The latter are two glycans that are usually restricted to the rough endoplasmic reticulum and to the perinuclear envelope. In this case GBP-1 was partially associated with endoplasmic reticulum (Figure 25D; GBP-1, red arrow; ConA, green arrow; colocalization, yellow arrows). Altogether this data indicated that IC-induced GBP-1 in HUVEC may associate partially with the endoplasmic reticulum.

2.4 Studies of GBP-1 association with detergent-resistant membranes

In a next step the nature of GBP-1 granular structures was investigated. In eukaryotic cells many types of vesicles are surrounded by coating proteins. Such proteins, together with sphingolipid-cholesterol rafts make the membranes of these vesicles insoluble in the detergent triton-X100 at 4 °C (Schekman*, et al.* 1996; Simons*, et al.* 1997; Helms*, et al.* 1998; Schekman 2002). An example of detergent-insoluble vesicles are the one originating from the endoplasmic reticulum and surrounded by coating type II (COPII) proteins (Tang*, et al.* 2000; Tang*, et al.* 2001). It has been shown that acylated proteins are associated with detergent-insoluble membrane fractions (Melkonian*, et al.* 1999). Prenylated proteins are largely excluded from detergent-resistant membrane fractions; nevertheless some prenylated proteins have been found also in detergent-insoluble fractions, for example Rap1 that is a monomeric GTPase that is closely related to Ras (Melkonian*, et al.* 1999).

GBP-1 has a CAAX isoprenylation motif (Figure 9) (Nantais*, et al.* 1996). GBP-1 prenylation could target GBP-1 to coated vesicles containing detergent-insoluble membranes and may cause the granular staining pattern of intracellular GBP-1. Therefore, the localization of GBP-1 in the detergent-soluble and/or insoluble fraction of HUVEC extracts was investigated. Cell membranes were extracted with the detergent triton-X100 from HUVEC incubated with IFN-γ (100 U/ml) for 24 h (Figure 26A). GBP-1 was found in both the detergent-soluble (Figure 26A, Sol.) and detergent-insoluble (Figure 26A, Ins.) fractions. Quantitative analysis of three Western blots indicated that in average 16.7 % of total GBP-1 protein localized in the detergent-insoluble fraction.

As a control that the separation of the detergent-soluble and -insoluble fractions was near to completion the Sec23 protein [a component of COPII coated vesicles (Tang*, et al.* 2000; Tang*, et al.* 2001)] was used. Western blot analysis of the same extracts described above

showed that Sec23, as expected, was localized exclusively in the detergent-insoluble fraction (Figure 26B).

These data suggested that GBP-1 is localized predominantly in the detergent-soluble fraction of proteins of the cytoplasm. However, a significant part of GBP-1 was present also in the detergent-insoluble fraction, which suggested a membrane association of the protein.

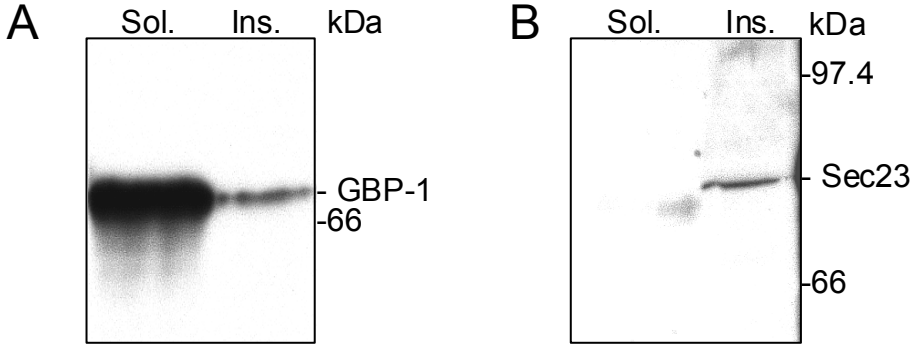


Figure 26: Detergent-extraction of GBP-1 protein from IFN-γ-stimulated HUVEC. *(**A**) HUVEC were incubated with IFN-γ (100 U/ml) for 24 h. Briefly, cell monolayers were incubated for 30 min on ice with lysis buffer containing 1 % triton-X100. Cells were scraped off and the lysate was centrifuged. The pellet was the insoluble fraction (Ins.). Proteins of the supernatant were precipitated with TCA (soluble fraction, Sol.). The GBP-1 content in these two fractions was investigated by Western blot analysis with MAb 1B1. (**B**) Western blot analysis of the same extracts as in **A** using an anti-Sec23 antibody.*

2.5 Studies of IFN-γ-induced GBP-1 expression in different cell types

GBP-1 mRNA expression has often been used as a marker to demonstrate IFN-γ activation of cells in culture (Ucer*, et al.* 1986; van Loon*, et al.* 1991; Tnani*, et al.* 1999; Yang*, et al.* 1999; Kumar*, et al.* 2001). However, GBP-1 protein expression has not been investigated in detail so far.

In order to investigate in which cell types *in vitro* GBP-1 expression was induced by IFN-γ at the protein level, a variety of eukaryotic cells including B-cells, T-cells, monocytes, primary mononuclear cells (PBMC), keratinocytes, primary fibroblasts and primary endothelial cells (HUVEC and dMVEC) were stimulated with IFN-γ (100 U/ml) for 16 h. GBP-1 protein expression was analyzed by Western blot. GBP-1 expression was up-regulated in all these cells with the exception of T-cells (Figure 27).

These results were in agreement with previous work on the expression of GBP-1 mRNA in different cells (Cheng*, et al.* 1983; Decker*, et al.* 1989; Saunders*, et al.* 1999).

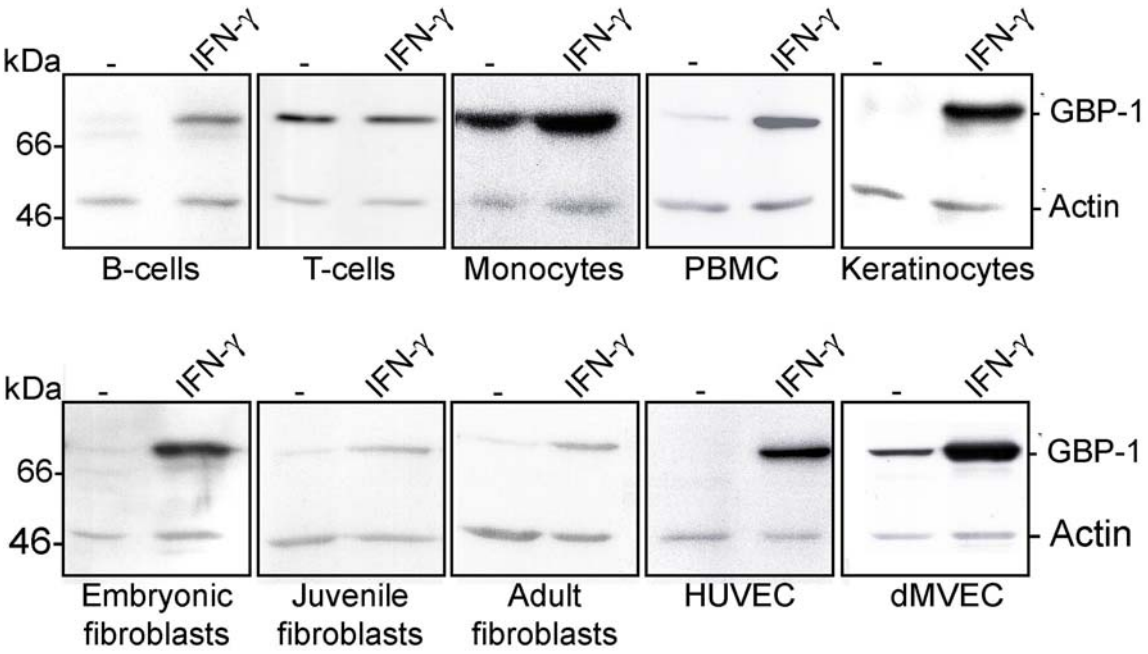


Figure 27: Western blot analysis of GBP-1 expression in different cell types stimulated with IFN-γ. *The different cell types indicated in the figure were incubated with IFN-γ (100 U/ml) or BSA (C) for 16 h. GBP-1 expression was analyzed by Western blot using MAb 1B1. Actin staining shows that similar amounts of protein extracts were loaded. From (Lubeseder-Martellato, et al. 2002). B-cells (Schlicht), T-cells (Hut 78), monocytes (U937), keratinocytes (HaCaT) and embryonic fibroblasts (HEF) were cell lines, whereas PBMC (primary mononuclear cells), the fibroblasts, HUVEC and dMVEC were primary cells.*

Summary of chapters 1 to 2 : IC-induced GBP-1 expression *in vitro*

In these first paragraphs GBP-1 expression was investigated at the protein level in HUVEC and dMVEC. IC (IFN-α, IFN-γ, IL-1α, IL-1β and TNF-α) induced GBP-1 expression. Notably, none of other chemokines or growth factors induced GBP-1 expression in HUVEC. In addition, GBP-1 subcellular localization in HUVEC was the same, irrespective whether IFN-γ, IL-1β or TNF-α were used to induce GBP-1 expression. Specifically the following results qualified GBP-1 as a molecular marker of IC-activated EC.

(1) It has been shown that stimulation of EC with IC, caused a stable GBP-1 expression. In addition, GBP-1 expression could be enhanced in HUVEC already expressing GBP-1.

(2) It has also been shown that pre-incubation of EC with AGF did not affect IC-induced GBP-1 expression, both in HUVEC and in dMVEC. Therefore, EC that are activated by IC express GBP-1 independently of a previous stimulation.

(3) Most importantly, AGF applied simultaneously with IC inhibit IC-induced GBP-1 expression. Therefore, GBP-1 expression reflects the relative concentrations of IC and AGF hat are active on EC.

Altogether this data qualify GBP-1 as a molecular marker for IC-activated EC *in vitro*.

3 Characterization of IC activation of EC *in vivo*

GBP-1 expression could be induced by IFN-γ in many different cells *in vitro* (see Figure 27). At present, no studies on GBP-1 expression *in vivo* were performed, due to the lack of specific antibodies. In order to investigate which cells may express GBP-1 in human tissues an immunohistochemical analysis of GBP-1 expression in paraffin-embedded human tissue was performed.

3.1 Expression of GBP-1 in normal human tissue

Immunohistochemical analysis of human tissue sections of spleen, uterus, lung and heart with MAb 1B1 demonstrated that GBP-1 was highly associated with endothelial cells (Figures 28A-D, black arrows). MAb 6F12 that recognized GBP-2 in addition to GBP-1 (see Results, paragraph **2.1** and Figure 13) produced similar staining patterns as compared to MAb 1B1 [compare Figure 28A (MAb 1B1) and Figure 28I (MAb 6F12)]. This indicated that GBP-1 is the major isoform expressed in EC.

In addition to EC, GBP-1 expression was detected in mononuclear cells in the bladder, lung (Figure 28C, brown arrows), stomach, colon and liver and in the epithelium in prostate, lung, colon, stomach and thyroid as assessed by morphological analysis (summarized in Table 3). Notably, GBP-1 was not detected in the skin (Figures 28E and 28F, negative vessels are indicated by a white arrow).

Control stainings with a primary antibody, which had been pre-adsorbed with GBP-1-His protein, did not produce any signals (Figure 28G, lung). In addition, no signals were obtained when the staining procedure was carried out without the primary antibody (data not shown) or with an isotype control antibody (Figure 28H, spleen).

The results obtained in the immunohistochemical analysis for GBP-1 expression in different tissues are summarized in Table 3.

In order to confirm EC association of GBP-1 in human tissues, double immunofluorescence studies were performed. Simultaneous detection of GBP-1 (Figure 29, left panels) and the EC-associated antigen CD31 (Figure 29, middle panels) in tissue sections of bladder (Figure 29A), endometrium (Figure 29B), heart (Figure 29C) and lung (Figure 29D) confirmed that GBP-1 was highly associated with EC in human tissues (Figure 29; GBP-1: green, left panel; CD31: red, middle panel; co-localization: yellow, right panel, white arrows).

All together these data indicated that GBP-1 expression was highly associated with endothelial cells in different human tissues *in vivo*.

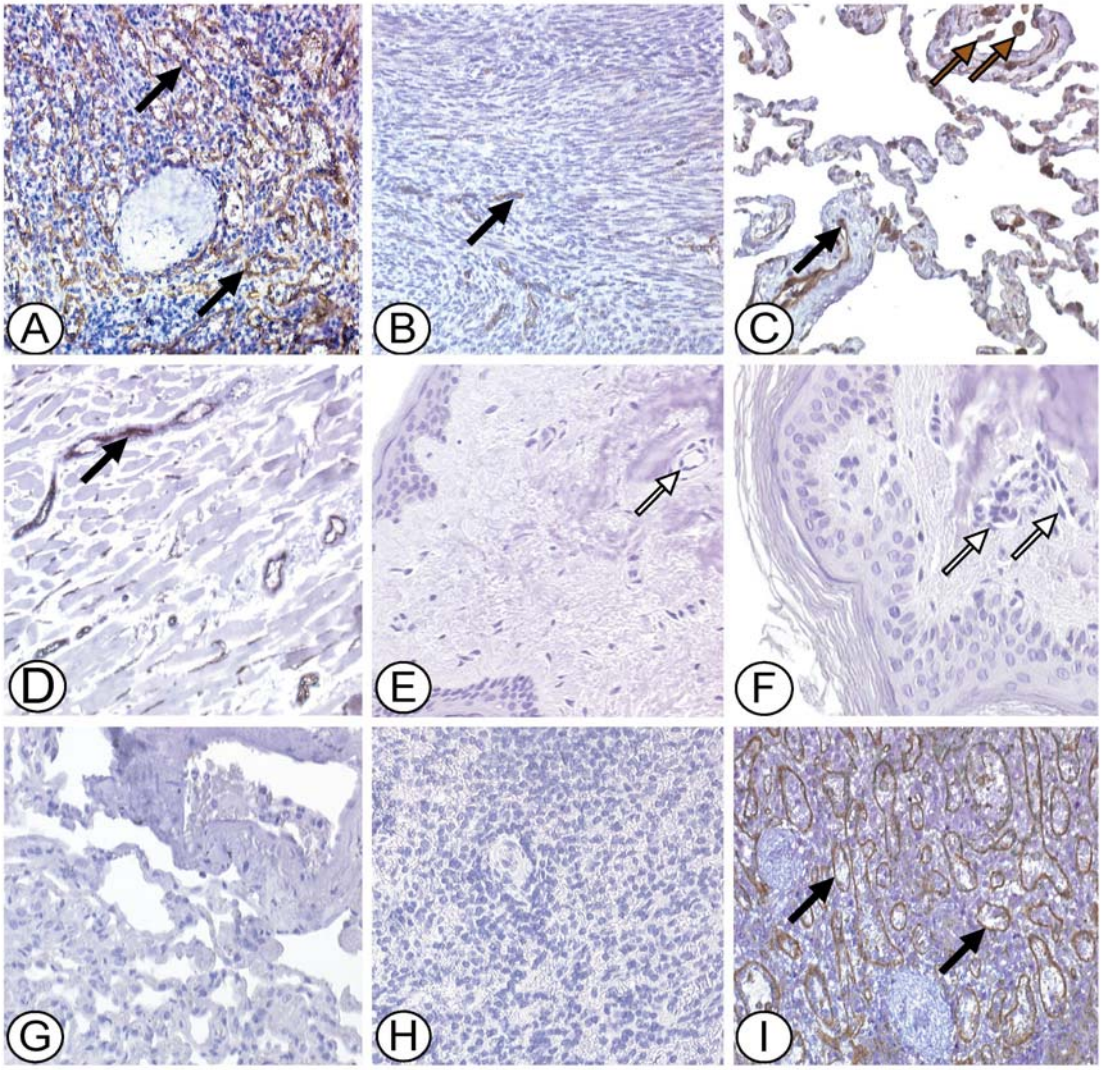


Figure 28: Immunohistochemical analysis of GBP-1 expression in various human tissues*. GBP-1 expression in paraffin-embedded sections of human spleen (**A** ,**I**), uterus (**B**), lung (**C**), heart (**D**) and skin (**E**,**G**) was detected using standard immunohistochemical staining with MAb 1B1 (**A-F**) and MAb 6F12 (**I**). In control stainings MAb 1B1 was pre-adsorbed with an excess (300 molar fold) of purified GBP-1-His (**G**, lung) or an isotype control antibody (**H**, spleen). Examples of GBP-1 positive (black arrows) and negative (white arrows) vessels are indicated. Mononuclear cells expressing GBP-1 are indicated by brown arrows. Magnification: x 250. From (Lubeseder-Martellato et al. 2002).*

3.2 GBP-1 expression in EC in diseases of the skin with a high-inflammatory component

Of note, GBP-1 was not expressed in healthy normal skin tissues (Figures 28E and 28F). In order to determine whether GBP-1 expression may be induced by IC in EC *in vivo*, GBP-1 expression was investigated in different skin diseases with a prominent inflammatory

component. AIDS-associated Kaposi's sarcoma (KS) is a neoplasm of vascular origin which expresses high levels of IC (Brooks 1986; Stürzl *et al.* 1995; Fiorelli *et al.* 1998; Ensoli *et al.* 2000; Guenzi *et al.* 2001; Stürzl *et al.* 2001). Adverse drug reactions and psoriasis are both characterized by a local inflammatory response involving infiltration of inflammatory cells, into the tissue and resulting in a local increase of IC concentration (Gomi*, et al.* 1991; Kapp 1993; Chodorowska 1999; Ackermann*, et al.* 1999; Yawalkar*, et al.* 2000).

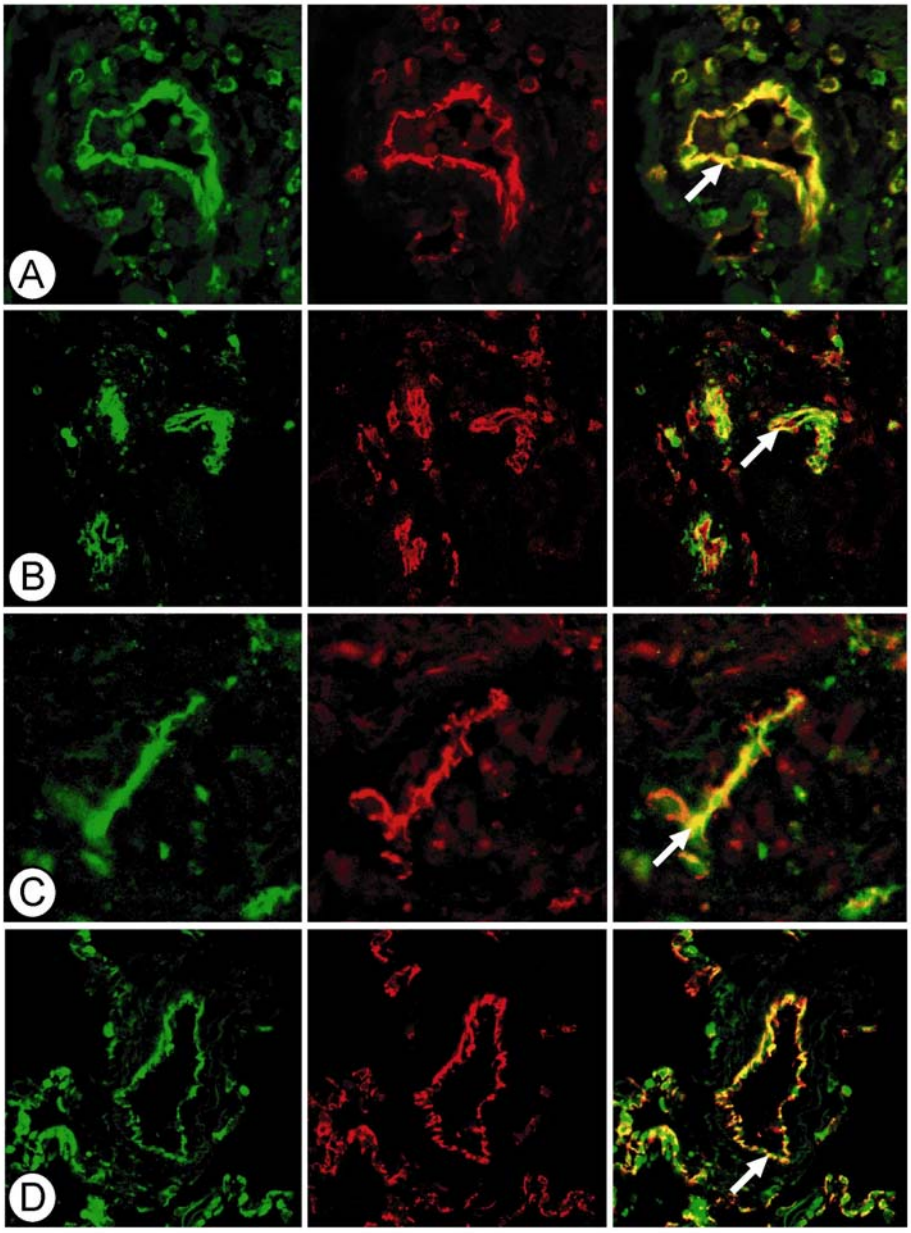


***Figure 29**: **Double immunofluorescence staining of GBP-1 and CD31 in human tissues**. Indirect immunofluorescence staining of tissue sections of bladder (**A**), endometrium (**B**), heart (**C**) and lung (**D**) for GBP-1 (using MAb 1B1) (green, left panels) and the endothelial cell associated antigen CD31 (red, middle panels). Merging of the two pictures (right panels) shows co-localization of GBP-1 and CD31 (yellow, white arrows). Magnification: x 400. From (Lubeseder-Martellato et al. 2002).*

Table 3**: **GBP-1 expression in different human tissues.

Tissue	n	GBP-1 positive vessels	Additional GBP-1 positive cells
Spleen	5	+	-
Bladder	5	+	$+^{m}$
Testis	5	+	-
Prostate	5	+	$+^{e}$
Ovary	5	+	-
Endometrium	5	+	$+^{g}$
Uterus	5	+	-
Placenta	5	+	-
Lung	10	+	$+^{m,e}$
Heart	9	+	-
Colon	6	+	$+^{m,e}$
Stomach	10	+	$+^{m,e}$
Thyroid gland	6	+	$+^{e}$
Brain	3	+	-
Kidney	8	-	$+^{gt}$
Liver	6	-	$+^{m}$
Skin	9	-	-

n, number of samples; +, positive staining; -, no staining; m, mononuclear cells; g, glands; e, epithelium; gt, glomeruli and tubuli. From (Lubeseder-Martellato et al. 2002).

Indirect immunofluorescence studies were performed on paraffin-embedded sections from different patients affected with KS (n=37, Figures 30B and 30E), adverse drug reactions of the skin (n=2, Figure 30C) and psoriasis (n=3, Figure 30D). Notably, GBP-1 was detected in all of these inflammatory skin diseases and in Kaposi's sarcoma (Figures 30B-E, left panels). GBP-1 expression was detected in small size vessels in adverse drug reactions of the skin (Figure 30C), in intermediate size vessels in KS (Figures 30B and 30E) and in larger vessels in psoriasis (Figure 30D). In all specimens GBP-1 expression was restricted to single vessels (Figures 30B-E). This is in agreement with the locally restricted expression of IC that has been reported in all of these lesions (Gottlieb*, et al.* 1988; Kapp 1993; Stürzl*, et al.* 1995; Fiorelli*, et al.* 1998; Ackermann*, et al.* 1999; Hari*, et al.* 1999; Ensoli*, et al.* 2000; Guenzi*, et al.* 2001; Stürzl*, et al.* 2001). GBP-1 was not detected in healthy skin (Figure 30A, left panel, compare also Figures 28E and 28F). In addition, in diseased skin, GBP-1 was selectively expressed in EC as indicated by simultaneous detection of GBP-1 and CD31 (Figures 30A-D; GBP-1: green, left panel; CD31: red, middle panel; co-localization: yellow, right panel, white arrows).

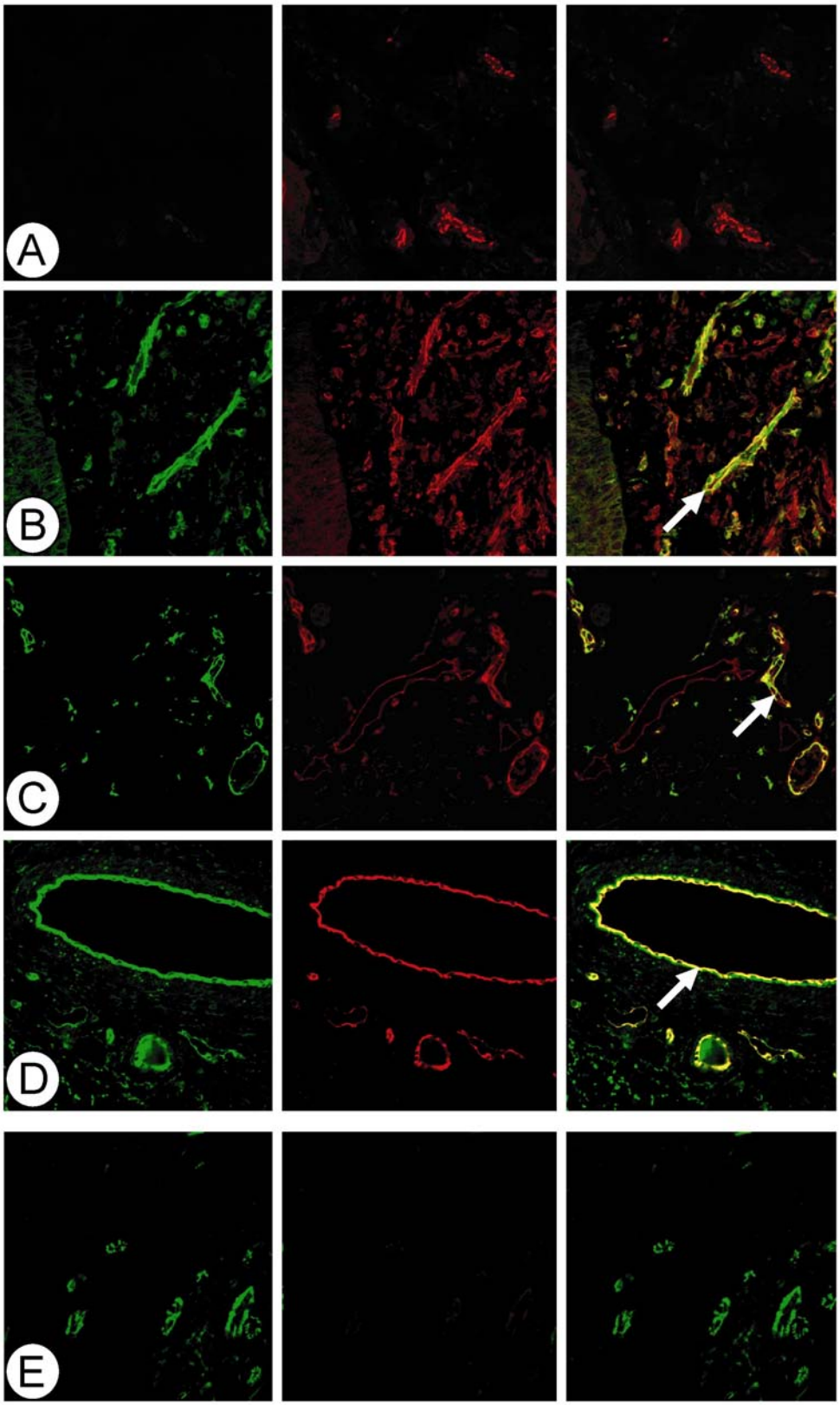


Figure 30: GBP-1 expression in vascular EC in skin diseases with a high inflammatory component. *Indirect immunofluorescence staining of tissue sections of healthy skin (**A**), Kaposi's sarcoma (**B**, **E**), adverse drug reaction of the skin (**C**) and psoriasis (**D**) for GBP-1 (green, left panels, **A-E**) and the EC associated antigen CD31 (red, middle panels, **A-D**). MAb 1B1 was used in **A**, **B** and **E**, MAb 6F12 was used in **C** and **D**. Merging of the two pictures (right panels) shows co-localization (yellow, white arrows). In (**E**) the mouse anti-CD31 antibody was omitted. Magnification **A, B, E**: x 250 and **C, D**: x 400. From (Lubeseder-Martellato et al. 2002).*

An anti-rat antibody coupled to the fluorochrome *Alexa488* and the anti-murine antibody coupled to the fluorochrome *Alexa546* were used as secondary antibodies for the detection of GBP-1 and CD31 respectively. The specificity of the cross absorbed secondary antibodies was demonstrated by the facts that GBP-1 negative skin sections did not show any green fluorescence of the anti-rat-*Alexa488* antibody (Figure 30A, left panel). Moreover, KS sections that were stained without the anti-CD31 antibody did not reveal any red fluorescence of the anti-murine-*Alexa546* antibody (Figure 30E, middle panel).
Altogether these data showed that GBP-1 expression is highly associated with EC *in vivo* and that GBP-1 is selectively upregulated by IC in inflammatory skin diseases. These findings suggested that GBP-1 characterizes the IC-activated phenotype of EC *in vivo*.

3.3 GBP-1 expression in Kaposi´s sarcoma

In order to confirm that GBP-1 expression in vessel endothelial cells of the skin is induced by IC, KS was used as *in vivo* model. As mentioned above KS is a neoplasm of vascular origin which expresses high levels of IC, in particular IFN-γ, IL-1β and TNF-α that play a crucial role in the early stages of KS (Brooks 1986; Ensoli*, et al.* 2000; Stürzl*, et al.* 2001). The main source of IC in KS lesion are infiltrating monocytes (Stürzl*, et al.* 1995; Fiorelli*, et al.* 1998).
Immunohistochemical studies for the simultaneous detection of GBP-1 and of monocytes demonstrated that GBP-1 is selectively expressed in vessels (Figures 31, black arrows) which are surrounded by numerous perivascular CD68-positive monocytes (Figures 31, red arrows).

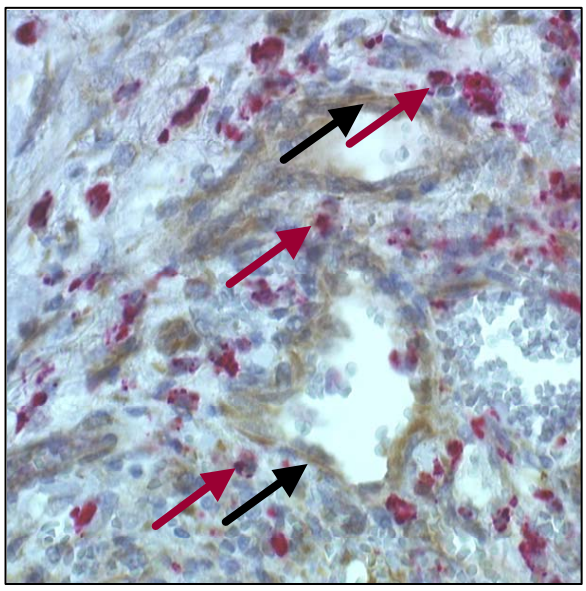


Figure 31: Immunohistological detection of GBP-1 and monocytes in KS tissue. *A KS section was stained using standard immunohistochemical techniques for the simultaneous detection of GBP-1 (brown, black arrows) and the monocytic marker protein CD68 (pink, red arrows). Magnification: x 670. From (Guenzi et al. 2001).*

In addition, GBP-1 expression was analyzed in KS tissue sections in which early (Figure 32, upper half) and late (Figure 32, lower half, white circle) developmental stages of KS were present simultaneously.

Early KS stages have been shown to express high concentrations of IC; by contrast in late KS stages an increased expression of AGF has been reported (Xerri*, et al.* 1991; Ensoli*, et al.* 1994; Stürzl*, et al.* 1995; Cornali*, et al.* 1996; Ensoli*, et al.* 2000; Stürzl*, et al.* 2001). Simultaneous detection of GBP-1 and CD31 indicated that in these sections GBP-1 was predominantly expressed in the areas presenting an early stage histology (Figure 32, upper half, yellow arrows). In contrast, in areas with nodular late stage histology, GBP-1 expression was clearly lower (Figure 32, lower half, white circle).

3.4 GBP-1 expression in non-proliferating vessel endothelial cells

GBP-1 has been shown to mediate the antiproliferative effect of IC on HUVEC *in vitro* (Guenzi*, et al.* 2001). In order to investigate whether GBP-1 may mediate the inhibition of EC proliferation by IC also *in vivo,* immunofluorescence stainings of KS tissue sections for the simultaneous detection of GBP-1, the endothelial cell-associated antigen CD31 and the proliferation-associated antigen Ki67 were performed.

Simultaneous detection of GBP-1 (Figures 33A and 33D, arrows; 33C and 33F, green) and Ki67 (Figures 33B 33E, arrows; 33C 33F, red) indicated that in no case GBP-1 and Ki67 (Figures 33B and 33E) were co-expressed in the same cell (Figures 33C and 33F). This demonstrated that GBP-1 is not expressed in proliferating EC. Specificity of Ki67 staining was demonstrated by the positive reaction of proliferating basal cells in the epidermis (Figure 33C, asterisk).

To further prove that GBP-1 is only expressed in non-proliferating, but not in proliferating EC within KS lesions, triple labeling experiments for the simultaneous detection of GBP-1, Ki67 and CD31 were performed in the tissue sections. CD31-positive EC surrounding tumor vessels were evenly distributed (Figures 33H, arrow and 33J, red cytoplasmic staining). In contrast, the highest number of GBP-1- (Figure 33G, arrows and 33J, green cytoplasmic staining) or Ki67- (Figure 33I, arrows and 33J, blue nuclear staining) positive EC were detected in different areas of the tissue section. Interestingly, areas with many Ki67-expressing EC (Figure 33J, upper part, blue arrows) revealed only few GBP-1-expressing EC, and *vice versa*, in areas with many GBP-1-expressing endothelial cells (Figure 33J, lower part, green arrows) only a few Ki67 positively stained cells were detected. Altogether, these

data strongly suggested that GBP-1 mediates the IC-induced inhibition of EC proliferation also *in vivo*.

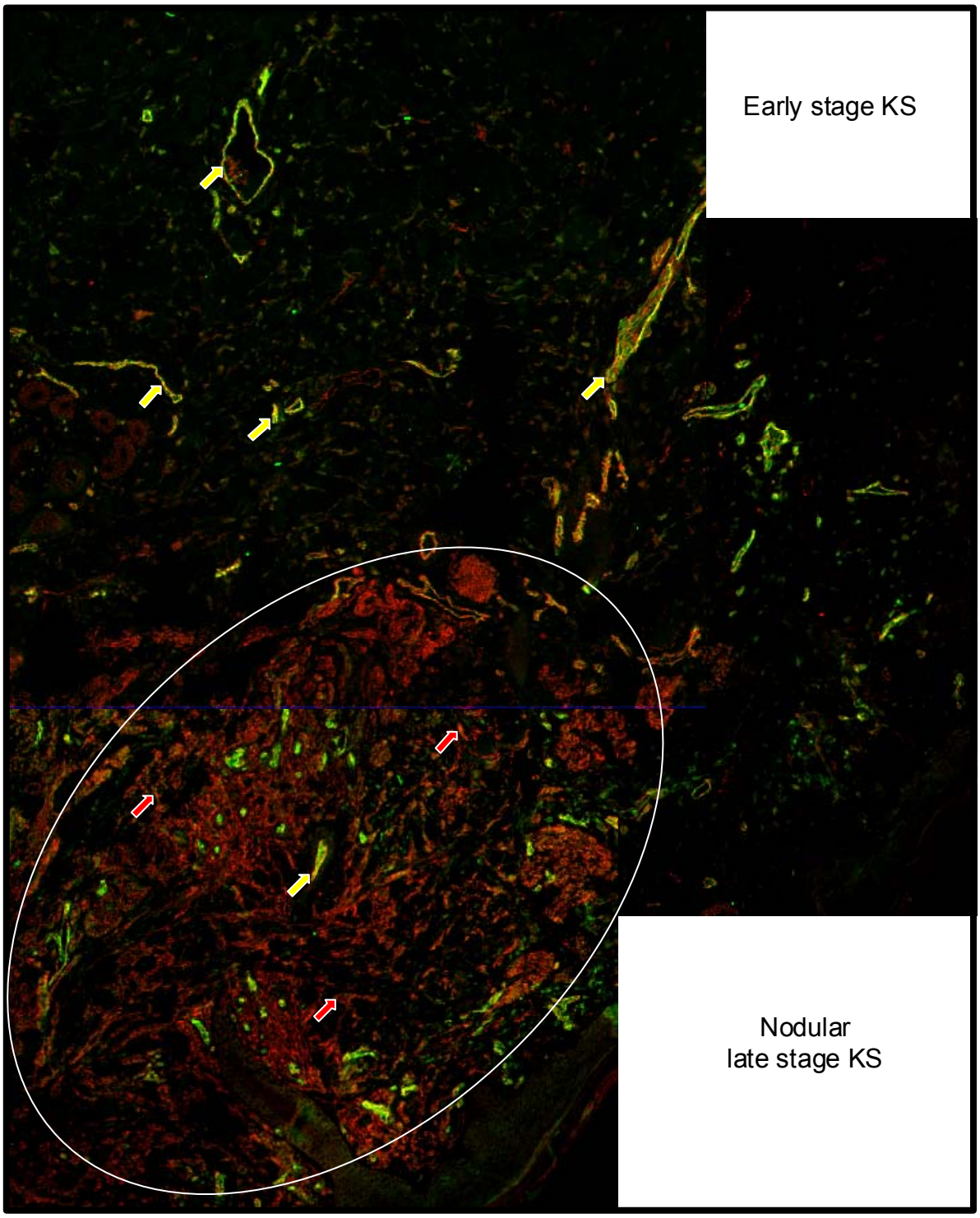


Figure 32: Overview of a KS lesion stained for GBP-1 and CD31. *A paraffin-embedded KS section presenting late stage (white circle) and early stage histology simultaneously was subjected to immunofluorescence for the detection of GBP-1 (green) and the EC-associated antigen CD31 (red). Red arrows: CD31 positive vessels, yellow arrows: CD31 positive vessels expressing GBP-1. For the detection of GBP-1 MAb 1B1 was used. This overview picture is a composite of three single pictures. Magnification: x 100.*

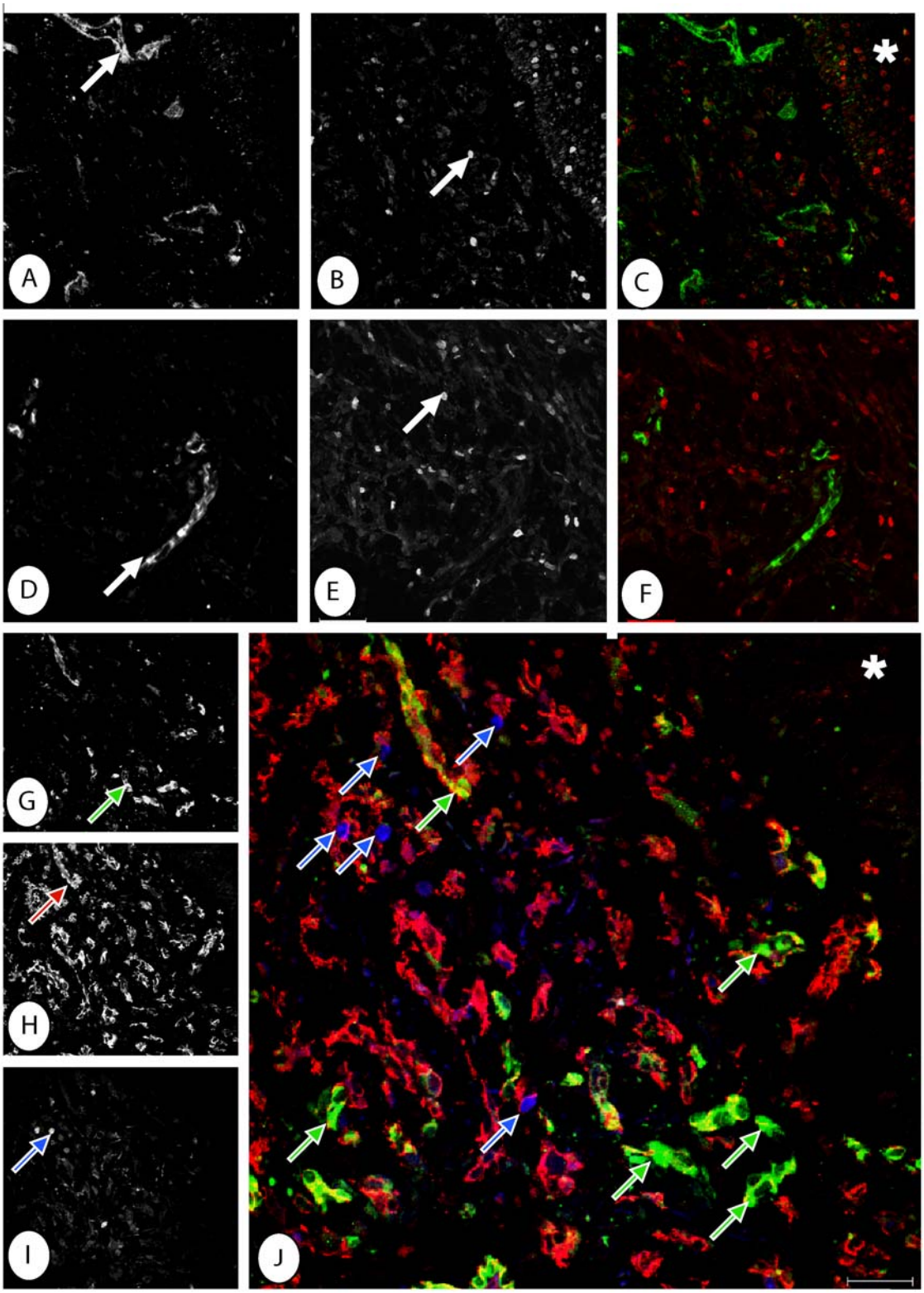


Figure 33: Indirect immunofluorescence staining of GBP-1, CD31 and Ki67 in KS lesions. *Paraffin-embedded KS sections were stained by immunofluorescence for the detection of GBP-1 (arrows, **A** and **D**) and Ki67 (arrows **B**, **E**) alone and in combination (GBP-1, green cytoplasmic staining; Ki67, red nuclear staining). Ki67 positive basal cells in the epidermis are labeled by an asterisk (**C** and **J**). Triple labeling experiment for the detection of GBP-1 (green arrow, **G**), CD31 (red arrow, **H**) and Ki67 (blue arrow, **I**) alone or in combination (CD31, red; GBP-1, green; Ki67, blue, **J**). Double positive EC are indicated: Ki67/CD31 (blue arrows) and GBP-1/CD31 (green arrows). Magnification: x 250. From (Guenzi et al. 2001)*

Summary of chapter 3 :

In the previous paragraphs the expression of GBP-1 *in vivo* has been investigated. It has been shown that GBP-1 expression was highly associated with EC in different human tissues *in vivo*, but that GBP-1 was not expressed in healthy normal skin tissues. Investigation of GBP-1 expression in different skin diseases with a prominent inflammatory component showed that GBP-1 expression is selectively upregulated by IC *in vivo*.

In particular in KS infiltrating monocytes produce high levels of IFN-γ, IL-1β and TNF-α. GBP-1 was selectively expressed in vessels that were surrounded by numerous perivascular CD68-positive monocytes. In addition, GBP-1 expression was analyzed in KS tissue sections in which early and late developmental stages of KS were present simultaneously. In these sections GBP-1 was predominantly expressed in the areas presenting an early stage histology which have been shown to express high concentrations of IC. By contrast, GBP-1 expression in these KS sections was clearly lower in areas with nodular late stage histology in which an increased expression of AGF has been reported.

Altogether these findings suggested that GBP-1 is a novel activation marker that characterizes the IC-activated phenotype of EC *in vivo*.

In addition, it was investigated whether GBP-1 may mediate the inhibition of EC proliferation by IC *in vivo.* For this purpose double and triple labeling experiments of KS tissue sections for the simultaneous detection of GBP-1, CD31 and the proliferation-associated antigen Ki67 were performed. Areas with many Ki67-expressing EC revealed only few GBP-1-expressing EC, and *vice versa*, in areas with many GBP-1-expressing EC only a few Ki67 positively stained cells were detected.

Altogether, these data strongly suggested that GBP-1 mediates the inhibition of EC proliferation by IC also *in vivo*.

4 Studies of GBP-1 secretion

GBP-1 has been shown to characterize the IC-activated phenotype of EC both *in vitro* and *in vivo* (see Results, paragraph **2.5** and **3.**). Moreover, GBP-1 localized partially in granular structures (see figure 30). This suggested that GBP-1 may be secreted. Therefore, the possibility that GBP-1 may be secreted and may a be soluble marker of IC activation of EC was investigated.

In a first step, GBP-1 was immunoprecipitated from the cell culture supernatant of HUVEC that have been stimulated with IFN-γ (100 U/ml) for 24 h with a polyclonal anti-GBP-1 antibody. Under these conditions GBP-1 expression was highly induced in these cells (Figure 34A, cell lysate). Notably, GBP-1 could also be detected in the cell culture supernatants of these cells after immunoprecipitation and subsequent Western blot analysis (Figure 34A, Sup, IFN-γ). In the immunoprecipitates also a smaller protein of about 47 kDa reacted with MAb 1B1. This protein was only detected in the cell culture supernatants of cells reated with IFN-γ (Figures 34A, Sup), but not in the cell lysate of the respective cells. GBP-1 was not in the supernatants of unstimulated cells (Figure 34A, Sup, -).

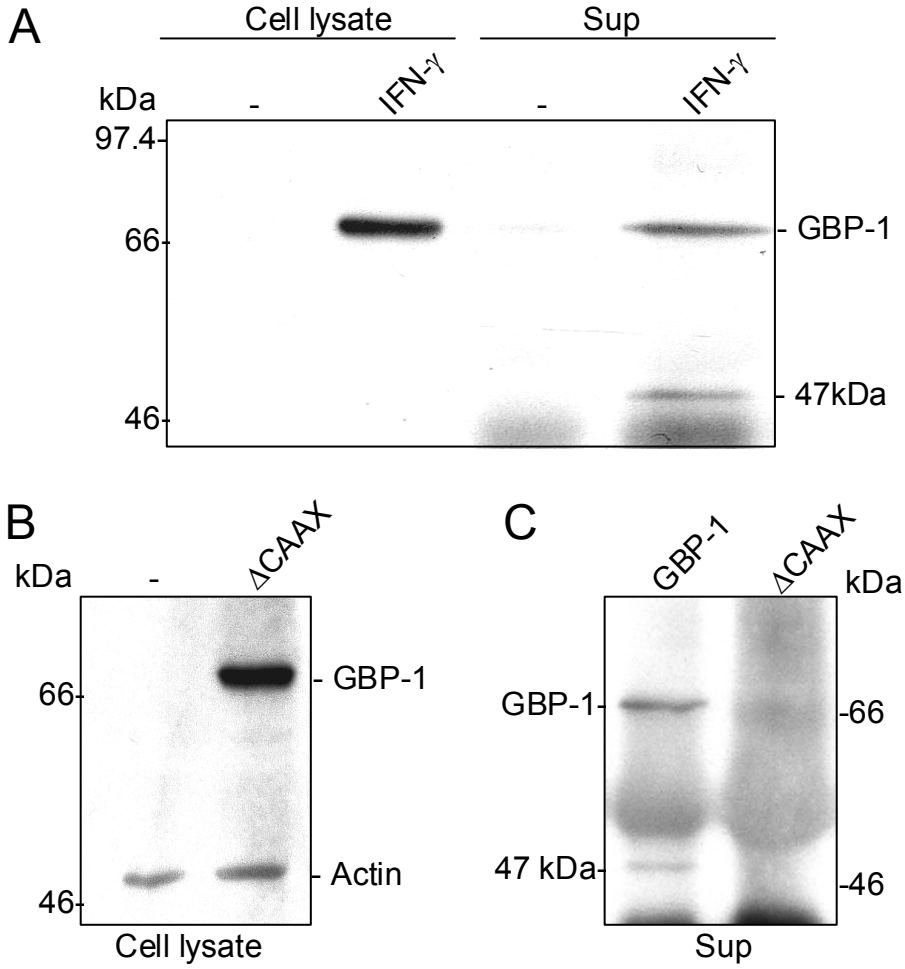


Figure 34: Detection of GBP-1 in cell culture supernatants of HUVEC. *(**A**) HUVEC were incubated for 24 h with IFN-γ (100 U/m) or with BSA (-). GBP-1 expression in the cell lysates was detected by Western blot analysis using MAb 1B1 (Cell lysate). GBP-1 was immunoprecipited with a polyclonal anti-GBP-1 antibody from the cell culture supernatants and then detected by Western blot using MAb 1B1 (Sup). (**B**) HUVEC were transduced with a retroviral vector that expressed a mutated GBP-1 without the CAAX motif (ΔCAAX) constitutively. Untreated wild type HUVEC were used as a control (-). Western blot analysis of the respective cells extracts was performed using MAb 1B1. Actin staining shows that comparable amounts of protein extracts were loaded. (**C**) HUVEC were transduced with a retroviral vector that expressed GBP-1 (GBP-1) or expressed the same deletion mutant of GBP-1 (ΔCAAX) as in (**B**) constitutively. GBP-1 was immunoprecipited with a polyclonal anti-GBP-1 antibody from the cell culture supernatants and detected by Western blot using MAb 1B1.*

In order to investigate if GBP-1 secretion was dependent on IFN-γ stimulation, HUVEC were transduced with a retroviral vector for constitutive expression of GBP-1. Similarly as observed with IFN-γ stimulated cells, GBP-1 could be detected in the cell culture supernatant of transduced cells by immunoprecipitation with a polyclonal anti-GBP-1 antibody and subsequent Western blot analysis; also in this case the 47 kDa fragment could be observed (Figure 34B, GBP-1).

Preliminary experiments were performed using HUVEC transduced with a retroviral vector for constitutive expression of a mutated GBP-1 without the CAAX motif (ΔCAAX) (Guenzi, *et al.* 2001). MAb 1B1 was able to recognize this mutant GBP-1 in Western blot (Figure 34B, ΔCAAX). Interestingly, this GBP-1 mutant could not be detected in the cell culture supernatant of these cells (Figure 34C, Sup, ΔCAAX, compare with GBP-1).

In a next step, GBP-1 was immunoprecipitated from the cell culture supernatants of HUVEC metabolically labeled with ^{35}S-Methionine. HUVEC were either incubated with IFN-γ (100 U/ml, 24 h) (Figure 35, IFN-γ) or transduced with a retroviral vector for constitutive expression of GBP-1 (Figure 35, GBP-1). GBP-1 was detected by autoradiography in the cell lysate of IFN-γ treated HUVEC (Figure 35A). Notably, GBP-1 was clearly detected in the supernatants of IFN-γ treated HUVEC and of HUVEC expressing GBP-1 constitutively (Figure 35B). No GBP-1 was detected in the cell lysate or in the supernatant of unstimulated control cells (Figure 35, -). This result confirmed the data obtained by immunoprecipitation followed by Western blot analysis.

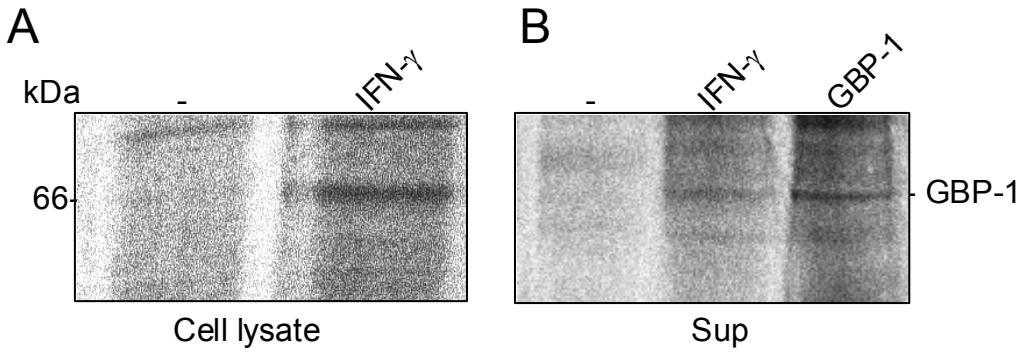


Figure 35: Detection of GBP-1 in cell lysates and cell culture supernatants of metabolically labeled HUVEC. *(**A** and **B**) HUVEC were grown for 16 h in low medium, washed twice with PBS and incubated in EGLM-2 medium (without methionine) for 1 h. Afterwards the cells were labeled with ^{35}S-Methionine (27.8 μCi/ml) and incubated with IFN-γ (100 U/ml) or with BSA (-) for 24 h. Alternatively, HUVEC were transduced with a retroviral vector that expressed GBP-1 constitutively (GBP-1) before labeling with ^{35}S-Methionine. (**A**) Intracellular GBP-1 was analyzed by SDS-PAGE of the cell extracts and subsequent autoradiography (Cell lysate). (**B**) GBP-1 from the cell culture supernatants (Sup) was immunoprecipited with a polyclonal anti-GBP-1 antibody. After separation by SDS-PAGE GBP-1 was detected by autoradiography.*

4.1 Studies of GBP-1 secreted by HUVEC in the absence of cell death

In a next step it was investigated whether the release of GBP-1 into the cell culture supernatant may be due to cell death the following experiments were performed. The unspecific exit of proteins from the cells can be verified by the presence of non-secreted abundant, cytosolic proteins in the cell culture supernatants.

Therefore, the presence of the abundant cytosolic protein glyceraldehyd phosphate dehydrogenase (GAPDH) was investigated in the cell culture supernatant of HUVEC treated with IFN-γ (100 U/ml) or BSA for 24 h. Immunoprecipitation experiments followed by Western blot analysis showed that GAPDH was present in high concentrations in the cell lysate of these cells (Figure 36A, Cell lysate). In contrast, GAPDH could neither be detected in the cell culture supernatants of untreated (Figure 36A, -) nor of IFN-γ-treated cells (Figure 36A, IFN-γ).

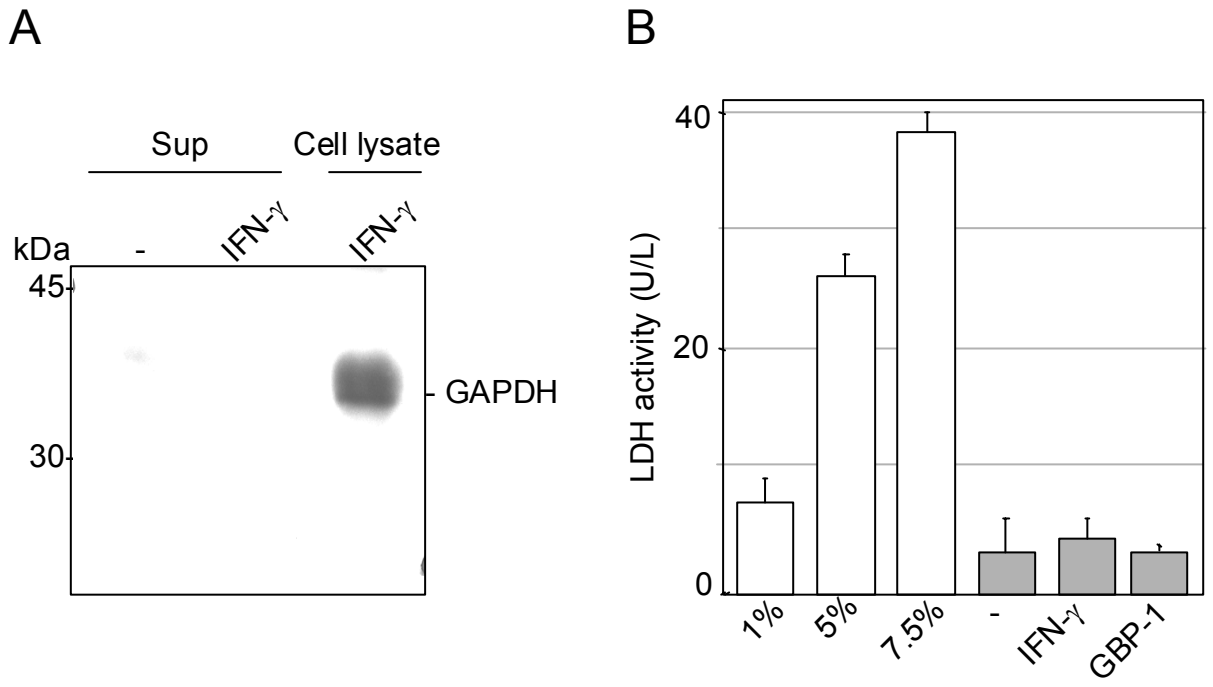


Figure 36: Detection of GAPDH and LDH in cell culture supernatants. *(**A**) Immunoprecipitation and subsequent Western blot analysis of GAPDH of cell lysates and in of cell culture supernatant (Sup) of HUVEC incubated with IFN-γ (100 U/ml) or with BSA (-) for 24. (**B**) Lactate dehydrogenase (LDH) activity assay of the cell culture supernatants of HUVEC either unstimulated (-), stimulated with IFN-γ (100 U/ml, 24 h) or transduced with a retroviral vector for the constitutive expression of GBP-1 (GBP-1). LDH activity of a standard was measured in order to correlate the result of the LDH activity assay with cell viability. The standard contained HUVEC lysed by freezing and thawing in concentrations of 1 %, 5 % or 7.5 % of the cell numbers used for in the stimulation described above.*

In an additional experiment, the presence of lactate dehydrogenase (LDH) in the cell culture supernatants was detected by measuring LDH activity. This method is considered as the most sensitive approach to detect alterations in cell permeability and non-specific release of intracellular proteins (Rubartelli*, et al.* 1990; Ensoli*, et al.* 1993; Chang*, et al.* 1997). LDH activity was analyzed in the cell culture supernatants of HUVEC treated with IFN-γ (100 U/ml) or BSA for 24 h (Figure 36B). LDH activity in the respective cell culture supernatants showed only a very low activity (Figure 36B, gray bars). LDH activity was not significantly increased in IFN-γ-treated HUVEC (Figure 36B, gray bar, IFN-γ) or in HUVEC transduced with a retroviral vector for the constitutive expression of GBP-1 (Figure 36B, gray bar, GBP-1) as compared to untreated cells (Figure 36B, gray bar, -). The standard used to correlate LDH activity with cell viability contained 1 %, 5 % or 7.5 %, of the cell number of HUVEC used for in the stimulation described above lysed by freezing and thawing. Comparison of LDH activity of the above described cell culture supernatants with the standard (Figure 36B, white bars) indicated that less than 1 % of these HUVEC had a damaged plasma membrane that may cause an unspecific exit of proteins.

In a final experiment, HUVEC treated with IFN-γ (100 U/ml) or BSA for 24 h were stained with Dead-Red, a membrane-impermeant dye that stains only cells with damaged plasma membranes (Figure 37A, red cells, arrows). In agreement with the LDH-analysis, less then 2 % of the HUVEC exhibited a damaged plasma membrane, irrespectively of IFN-γ treatment (Figure 37B, black bars).

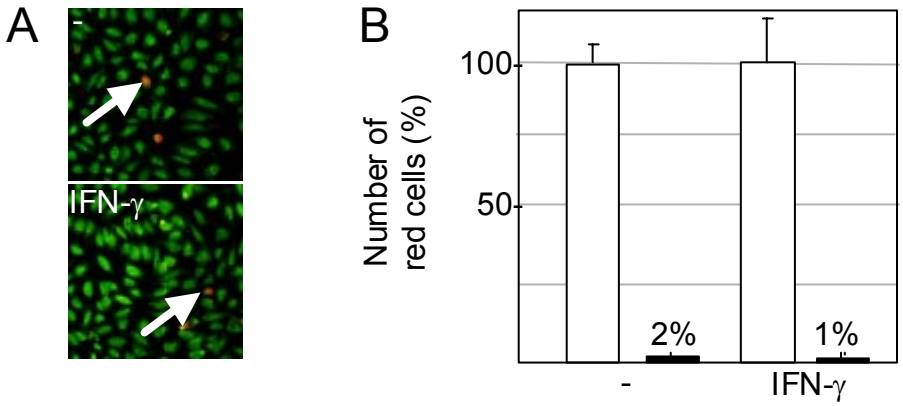


Figure 37: Detection of cell death in IFN-γ-treated HUVEC. *(**A** and **B**) HUVEC were incubated with IFN-γ (100 U/ml) or with BSA (-) or 24 h. (**A**) HUVEC were stained with the membrane-impermeant dye Dead-Red (red) and with the membrane permeant dye Syto10 (green). Under this conditions living cells are green, whereas dead cells or cells with altered membrane permeability are stained in red (arrows). (**B**) Cells from three optical fields like in **A** were counted. White bars = total number of cells. Black bars = relative amount of cells with altered membrane permeability.*

All of these data indicated that HUVEC expressing GBP-1 constitutively or after IFN-γ treatment are able to release it actively in the cell culture supernatant, independently from cell death.

In order to determine the relative amount of secreted GBP-1 in comparison to intracellular GBP-1, the concentrations of intracellular and extracellular GBP-1 were determined in HUVEC incubated with IFN-γ (100 U/ml) for 24 h. Proteins in the cell culture supernatant were concentrated by TCA precipitation. GBP-1 was detected in the precipitate by Western blot analysis. A quantitative determination by comparison of the signal intensities to the signals obtained with defined amounts of purified recombinant GBP-1-His was performed. The amount of the precipitated GBP-1 was normalized to 1 mg of total cell lysate. By this method it could be determined that the cell culture supernatants of these cells contain 53 ng of GBP-1 and the cell lysates of the same cells contain 555 ng of GBP-1. This indicated that about 10 % of total intracellular GBP-1 was secreted from IFN-γ-treated HUVEC into the cell culture supernatant. In addition, these results indicated that the amount of GBP-1 found in the cell culture supernatants may not be explained by the percentage of dead cells. Therefore, these data suggested that extracellular GBP-1 is not due to the release from dead cells but is predominantly secreted from intact cells.

Altogether these results, demonstrated that GBP-1 is released into the cell culture supernatant of IFN-γ-treated HUVEC or from HUVEC expressing GBP-1 constitutively. This release is not due to alterated membrane permeability of the cells, apoptosis or necrosis of the cells.

4.2 Development of an anti-GBP-1 enzyme-linked immunoadsorbent assay (ELISA)

In order to generate a tool for the quantification of GBP-1 in solution, a three-step sandwich-ELISA for the rapid detection of soluble GBP-1 was developed. Briefly, ELISA plates were first coated with anti GBP-1 MAb 1B1 hybridoma supernatants diluted 1:5. Second, after addition of the samples, a rabbit anti-GBP-1 antibody was added. Third, an alkaline phosphatase (AP)-conjugated anti-rabbit antibody was added. P-nitrophenyl phosphate (PNPP) was used as a substrate for the AP. The absorbance was measured at 405 nm (A_{405}). For evaluation of this method recombinant purified GBP-1-His protein was used as a standard. As a control for specificity of the ELISA, increasing concentrations of BSA were used. As shown in Figure 38, the A_{405} increased in a concentration-dependent manner in the presence of GBP-1-His (gray bars), by contrast the A_{405} did not increase with increasing concentration of BSA (white bars).

In order to test whether the His-tag affected the sensitivity of the ELISA, the concentrations of serially diluted recombinant GBP-1-His and His-GBP-1 standards were measured by the ELISA (Figure 38B). No significant difference was observed in the A_{405} measured from the same concentrations of GBP-1-His and His-GBP-1 (Figure 38B). This indicated that the sensitivity of the ELISA was not affected by the His-tag.

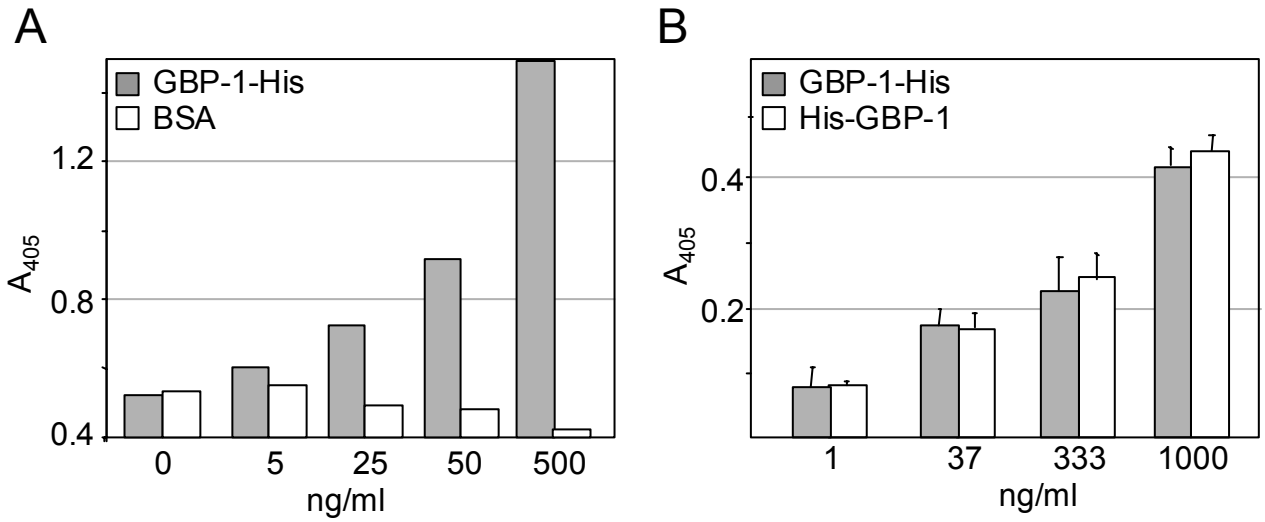


Figure 38: Detection of recombinant GBP-1 by ELISA. *(**A**) A serial dilution of purified recombinant GBP-1-His diluted in PBS in the indicated concentrations was analyzed by ELISA (gray bars). The same concentrations of BSA in PBS were used as a control (white bars). The absorbance was measured at 405 nm (A_{405}). The A_{405} increased in a concentration-dependent manner in the presence of GBP-1-His. By contrast the A_{405} did not increase with increasing concentration of BSA. (**B**) A serial dilution of purified recombinant GBP-1-His (gray bars) and His-GBP-1 (white bars) diluted in PBS at the indicated concentrations was analyzed by ELISA. Similar results were obtained independently whether the his tag was at the N-terminal or at the C-terminal end of recombinant GBP-1.*

With this ELISA increased concentrations of GBP-1 could be detected in the cell culture supernatant of HUVEC treated with IFN-γ (100 U/ml) for 24 h (Figure 39A). This result was well in agreement with the result obtained by immunoprecipitation (Figure 39B) of GBP-1 from the same cell culture supernatant: GBP-1 could be detected only in the cell culture supernatant of IFN-γ-treated (100 U/ml, 24 h) HUVEC, but not in the cell culture supernatant of untreated cells (-) (Figure 39). In agreement with the amount of secreted GBP-1 determined by TCA precipitation, the amount of secreted GBP-1 determined by ELISA was in average 50 ng GBP-1/mg of total protein content (Figure 39A).

In summary, the development of this ELISA allowed the quantitative determination of soluble GBP-1 in different samples.

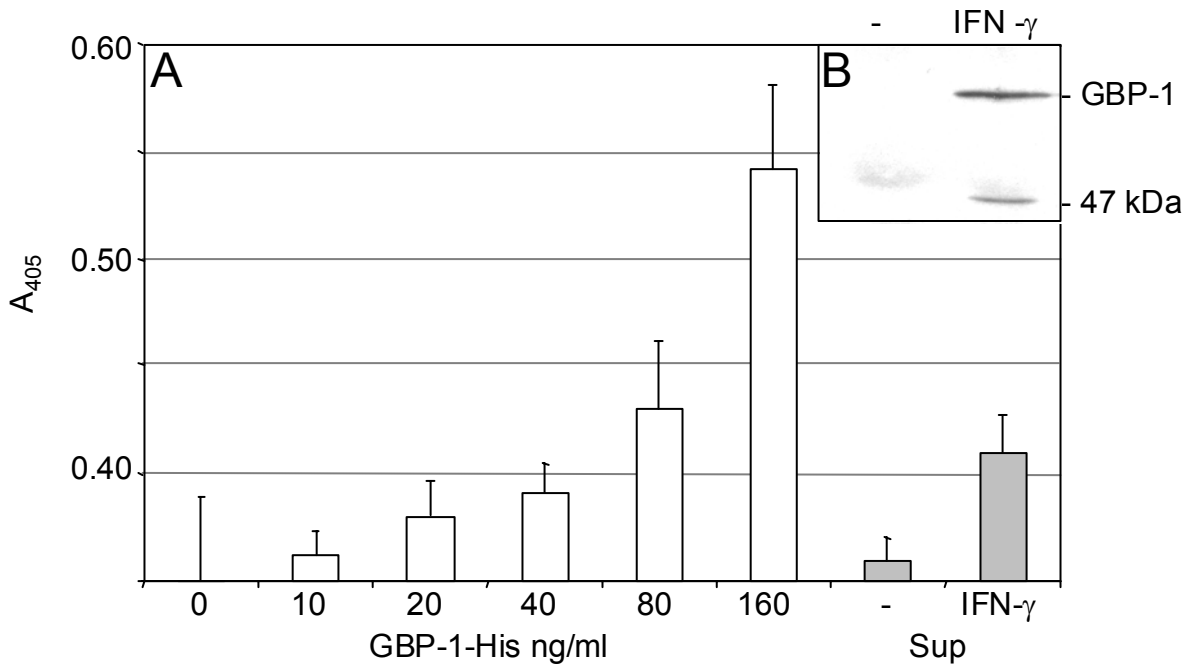


Figure 39: Detection of GBP-1 in the cell culture supernatant of HUVEC treated with IFN-γ. *(**A**) HUVEC were incubated with IFN-γ (100 U/ml) or with BSA (-) for 24 h. The concentration of GBP-1 in the cell culture supernatant (Sup, gray bars) was measured by ELISA. A dilution series of recombinant purified GBP-1-His was used a standard (white bars). The absorbance was measured at 405 nm (A_{405}). (**B**) GBP-1 was immunoprecipitated from the same cell culture supernatant of (**A**) using a polyclonal anti GBP-1 antibody. After SDS-PAGE the immunoprecipitate was analyzed by Western blot using MAb 1B1. GBP-1 and a protein of 47 kDa could be detected.*

4.3 Modulation of GBP-1 secretion

In order to investigate the pathway of GBP-1 secretion in IFN-γ-treated HUVEC, the effects of different culture conditions and different pharmacological agents on GBP-1 secretion were analyzed.

First, a decreased incubation temperature to 20 °C during IFN-γ (100 U/ml, 24 h) stimulation of HUVEC abrogated GBP-1 secretion as assessed by immunoprecipitation of GBP-1 from the cell culture supernatant (Figure 40A, compare IFN-γ at 20 °C with IFN-γ at 37 °C). This result was confirmed by quantification of GBP-1 in the same cell culture supernatant by ELISA (Figure 42). Incubation of IFN-γ-treated HUVEC at 20 °C did only slightly affect the amount of intracellular GBP-1, as assessed by Western blot analysis of the cell lysate (Figure 40B, upper panel). In addition, subcellular localization of GBP-1 was not affected by the decreased incubation temperature (Figure 40B, inserts). This indicated that GBP-1 may be released *via* an active and energy-dependent process.

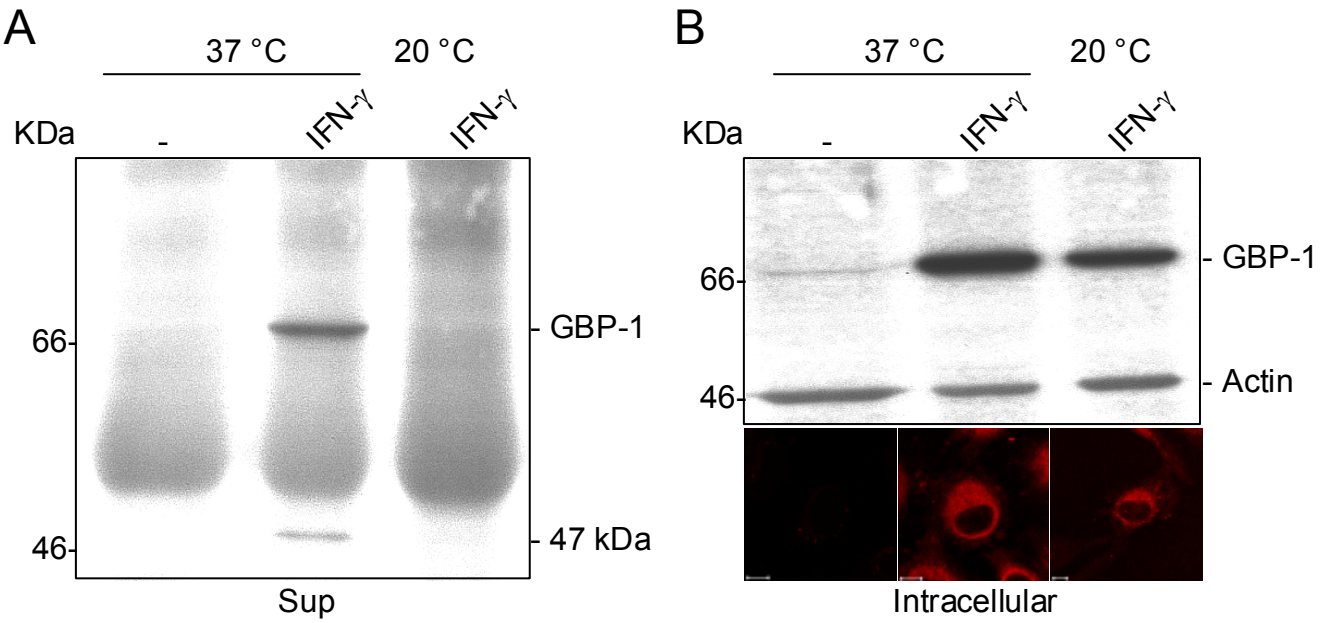


Figure 40: Analysis of the effect of the incubation at 20 °C on GBP-1 secretion. *HUVEC were incubated with IFN-γ (100 U/ml) or with BSA (-) for 24 h at 37 °C or 20 °C respectively. (**A**) Western blot analysis of GBP-1 that was immunoprecipited with an anti-GBP-1 polyclonal antibody from the cell culture supernatant (Sup) of the same cells as in **B**. (**B**) Western blot analysis (upper panel) and immunofluorescence staining (lower panel) of intracellular GBP-1. Actin staining shows that comparable amounts of protein extracts were loaded. In both cases MAb 1B1 was used. In all immunofluorescence pictures scale bar = 10 μm.*

Second, brefeldin A (BFA), a well established inhibitor of the classical secretory pathway was added to the cells 2 h prior to IFN-γ (100 U/ml, 24 h) stimulation (Misumi, *et al.* 1986; Rubartelli, *et al.* 1990; Jackson, *et al.* 1995; Chang, *et al.* 1997; Soderberg, *et al.* 2000; Taraboletti, *et al.* 2000; Hisadome, *et al.* 2002). In the classical secretion pathway proteins are targeted to the endoplasmic reticulum, then to the Golgi apparatus and finally to secretory vesicles [reviewed in (Harter, *et al.* 2000; Allan, *et al.* 2002; Joiner, *et al.* 2002) see also (Schekman, *et al.* 1996; Helms, *et al.* 1998; Schekman 1998; Kirchhausen 2000; Schekman 2002)]. The matrix metallo-proteinase-1 (MMP-1) follows the classical secretion pathway (Taraboletti, *et al.* 2000). Therefore, MMP-1 was used as a control for the effectivity of BFA treatment. MMP-1 expression was induced in HUVEC by treatment with angiogenic growth factors (AGF, VEGF and bFGF, 10 ng/ml each) for 16 h (Figure 41A). Immunoprecipitation of MMP-1 from the cell culture supernatant showed that MMP-1 is constitutively secreted irrespectively of the stimulation (Figure 41B, - and AGF). However, after treatment with BFA (1 μg/ml, 2 h before AGF stimulation), the amount of extracellular MMP-1 was significantly reduced (Figure 41B, BFA + AGF). In addition, MMP-1 accumulated in the cell (Figure 41A, BFA + AGF).

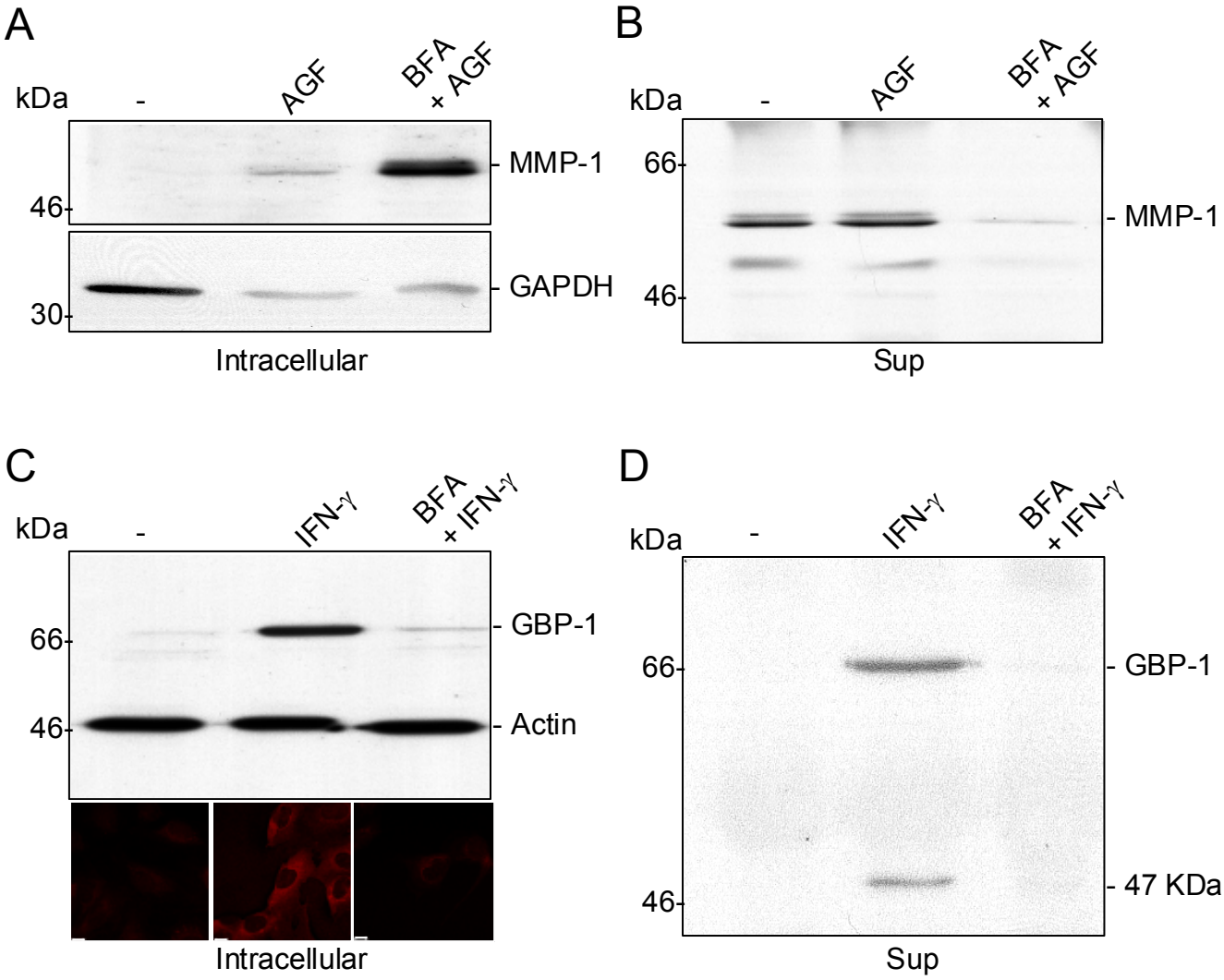


Figure 41: Modulation of MMP-1 and GBP-1 secretion in HUVEC using brefeldin A (BFA). *(**A** and **B**) Cells were incubated with AGF (VEGF and bFGF, 10 ng/ml each), with BSA (-) or with AGF together with BFA (1 μg/ml, 2 h before addition of AGF) for 16 h (BFA + AGF). (**A**) Western blot analysis for intracellular MMP-1 expression. GAPDH staining shows that in the control more of the protein extract was loaded, nevertheless no MMP-1 is visible. (**B**) Western blot analysis of MMP-1 immunoprecipited from the cell culture supernatant of the same cells. (**C** and **D**) Cells were incubated with IFN-γ (100 U/ml), with BSA (-) or with IFN-γ together with BFA (1 μg/ml, 2 h before addition of IFN-γ) for 24 h (BFA + IFN-γ). (**C**), upper panel: Western blot analysis of intracellular GBP-1. (**C**), lower panel: immunofluorescence staining of GBP-1. In both cases MAb 1B1 was used. Actin staining shows that equal amounts of protein extracts were loaded. In all immunofluorescence pictures scale bar = 10 μm. (**D**) Western blot analysis of GBP-1 immunoprecipited with an anti-GBP-1 polyclonal antibody from the cell culture supernatant of the same cells as in (**C**).*

Interestingly in HUVEC the induction of GBP-1 expression by IFN-γ (Figure 41C, IFN-γ) was almost fully inhibited by treatment with BFA (1 μg/ml, 2 h before IFN-γ stimulation) as shown by Western blot analysis and immunofluorescence staining of intracellular GBP-1 (Figure 41C, BFA+IFN-γ). Therefore, the reduction of GBP-1 in the cell culture supernatant (Figure 41B, right panel) could not be attributed to a possible inhibition of GBP-1 secretion by BFA.

Third, the effects of pharmacological agents (monensin, methylamine and verapamil) that are known to inhibit (**i**) the secretory function of the endoplasmic reticulum/Golgi apparatus, (**ii**) exocytosis and (**iii**) the multidrug resistance pathway were investigated on GBP-1 secretion (Rubartelli*, et al.* 1990; Jackson*, et al.* 1995). None of this substances affected membrane permeability as assessed by the LDH activity assay (data not shown) in HUVEC incubated with IFN-γ (100 U/ml, 24 h). Pre-treatment of HUVEC before IFN-γ (100 U/ml, 24 h) stimulation with monensin (1 nM, 1 h) [an inhibitor of the classical secretion pathway (Tartakoff 1983)] or with methylamine (1 nM, 1 h) [a drug able to modulate an alternative secretory pathway (Rubartelli *et al.* 1990)] increased GBP-1 secretion more then 3 fold (Figure 42). Pre-treatment (30 min) with verapamil (500 ng/ml) [an inhibitor of volume regulated anion channels and from the multidrug resistance pathway (Hisadome*, et al.* 2002)] increased GBP-1 secretion more then 2 fold (Figure 42). Methylamine and verapamil have also been shown to increase the secretion of aFGF (Jackson*, et al.* 1995). Therefore, the effects of these drugs on GBP-1 secretion were similar to their effect on aFGF secretion. This suggested that GBP-1 and aFGF may follow similar secretion pathways.

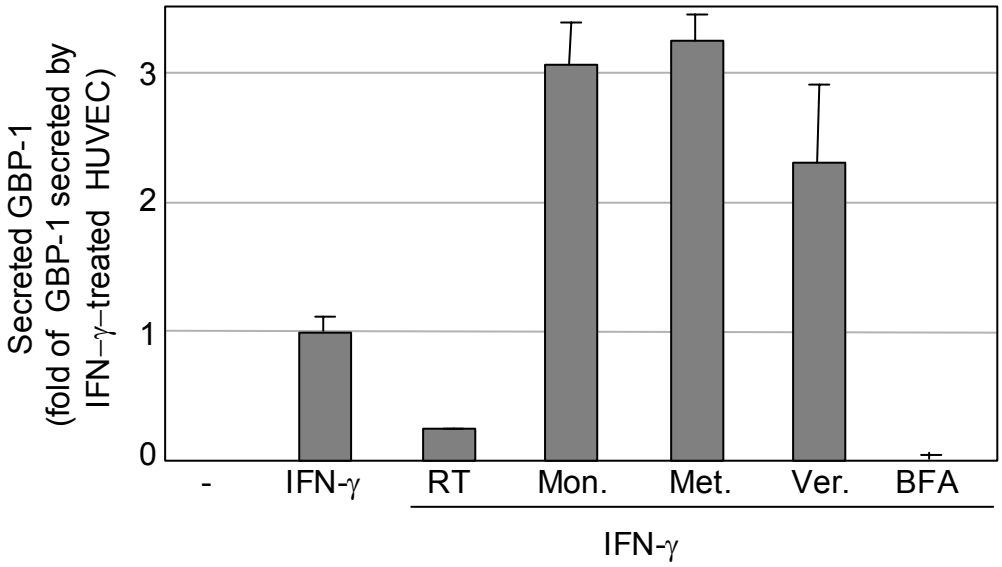


***Figure 42**: **Modulation of GBP-1 secretion using different pharmacological agents**. HUVEC were incubated in low medium and then incubated with BSA (-) or with IFN-γ (100 U/ml) for 24 h. For modulation of GBP-1 secretion HUVEC were either incubated with IFN-γ and incubated at room temperature (RT) or pre-incubated with monensin (Mon., 1 nM, 1 h), methylamine (Met., 1 nM, 1 h), verapamil (Ver., 500 ng/ml, 30 minutes) or BFA (1 μg/ml, 2 h). The amount of secreted GBP-1 per mg of total protein content was calculated from the amount determined by ELISA and/or TCA precipitation. The values obtained were normalized to the amount of GBP-1 secreted from IFN-γ-treated EC. The mean of at least three experiments (± SD) is shown.*

Fourth, decreasing serum concentrations have been shown to affect the secretion of proteins (Chang, *et al.* 1997; Rubartelli, *et al.* 1990). However, incubation of IFN-γ (100 U/ml)-stimulated HUVEC in low- (0.5% FBS) or full-medium (5% FBS) had no effect on GBP-1 secretion (data not shown).

In summary, these results indicated that GBP-1 is actively secreted through an energy-dependent mechanism, as the secretion could be inhibited by incubation of the cells at 20 °C. Analogy of the effects of monensin and methylamine on GBP-1 and aFGF release indicate that GBP-1 may be released via a signal peptide independent alternative pathway.

In a next step, it was investigated whether GBP-1 secretion is also activated by low physiological concentrations of IFN-γ (1 U/ml = 50 pg/ml) or by IFN-α.

HUVEC were treated with of IFN-γ (1 U/ml), IFN-α (100 U/ml) and with IFN-γ (100 U/ml) as a positive control for 24 h. In all cases GBP-1 expression was induced in the cells as assessed by Western blot analysis (Figure 43, cell lysates). Immunoprecipitation of GBP-1 in the respective cell culture supernatants and subsequent Western blot analysis showed that GBP-1 was also secreted by HUVEC under this conditions (Figure 43, Sup). Of note, also in this case in addition to GBP-1 the 47 kDa fragment could be observed in the cell culture supernatants of all stimulated HUVEC (Figure 43, 47 kDa). The same results as observed with HUVEC stimulated for 24 h were observed when HUVEC were stimulated for 48 h (data not shown), indicating the GBP-1 is stable in the cell culture supernatants.

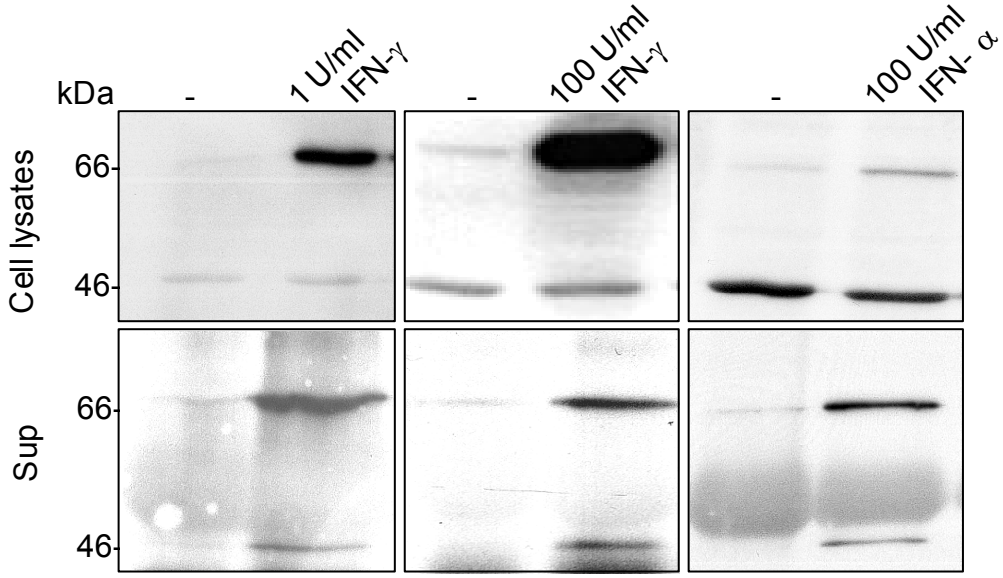


Figure 43: Stimulation of GBP-1 secretion by physiological concentrations of IFN-γ and by IFN-α. *HUVEC were incubated with BSA (-), with IFN-γ or with IFN-α at the indicated concentrations for 24 h. Upper panel: Western blot analysis of the cell lysates using MAb 1B1. Actin staining shows that comparable amounts of protein extracts are loaded. Lower panel: GBP-1 was immunoprecipited with a polyclonal anti-GBP-1 antibody from the cell culture supernatants (Sup) and detected by Western blot using MAb 1B1.*

Release of GBP-1 from cells stimulated with physiological concentrations IFN-γ was also quantitatively determined by ELISA. HUVEC were incubated with IFN-γ (1 U/ml) and with IFN-γ (100 U/ml) as a positive control for 24 h. The analysis indicated that the amount of GBP-1 secreted from HUVEC treated with 1 U/ml IFN-γ was only 25 % of the amount secreted from HUVEC treated with 100 U/ml of IFN-γ.
Altogether these data indicated that physiological concentrations of IFN-γ also induce GBP-1 secretion and that GBP-1 is also secreted by IFN-α stimulated HUVEC.

4.4 Studies of cell specific GBP-1 secretion

In order to test if GBP-1 secretion is endothelial cell specific, GBP-1 expression and secretion were examined in different adherent cell types including dMVEC, fibroblasts, and keratinocytes (Figure 44). In all of these cells GBP-1 expression could be induced by IFN-γ-treatment (Figure 44, upper panels; compare also Figure 16). After stimulation with IFN-γ (100 U/ml) for 24 h, GBP-1 was immunoprecipitated from the cell culture supernatants with a polyclonal anti-GBP-1 antibody and analyzed by Western blot. Interestingly, GBP-1 was only detected in the cell culture supernatants of dMVEC, but not in those of fibroblasts, or keratinocytes (Figure 44A, lower panels).
Determination of the GBP-1 concentration in the respective cell culture supernatants by ELISA confirmed that fibroblasts and keratinocytes did not secret detectable amounts of GBP-1 into the cell culture supernatant (Figure 44B). HUVEC secreted GBP-1 (about 50 ng/mg total protein content), (Figure 44B). The amount of secreted GBP-1 was calculated *per* mg of total protein content, which is a degree of the relative number of cell seeded
Altogether these data suggested that the capability to secrete GBP-1 may be a specific marker of endothelial cells.

4.5 Detection of GBP-1 in blood samples of patients

It has been shown that GBP-1 is a marker of IC-activated EC, in human tissues *in vivo* (see Paragraph **3**). Moreover, GBP-1 is secreted *in vitro* from IC-activated EC (see Paragraph **3.5**). Therefore, GBP-1 may be a serologically detectable marker of IC-activation of EC in patients. In a first step it was investigated whether GBP-1 can be detected in serum samples by ELISA and whether freezing of the samples reduced the detection sensitivity. To this goal purified recombinant His-GBP-1 was diluted in human serum and the respective concentrations were

measured by ELISA (Figure 45, gray bars). Parallel to the decreased amount of GBP-1 added, decreasing amounts of GBP-1 were detected by the ELISA. This indicated that GBP-1 can be detected in the serum by the ELISA method. In a next step the samples were subjected to repeated freezing-thawing. Subsequent detection of His-GBP-1 showed a similar concentration dependent decrease (Figure 45, white bars). This indicated that freezing of the serum samples does not impair the detection of GBP-1 in the samples. Therefore GBP-1 may be detected by ELISA in archived blood samples.

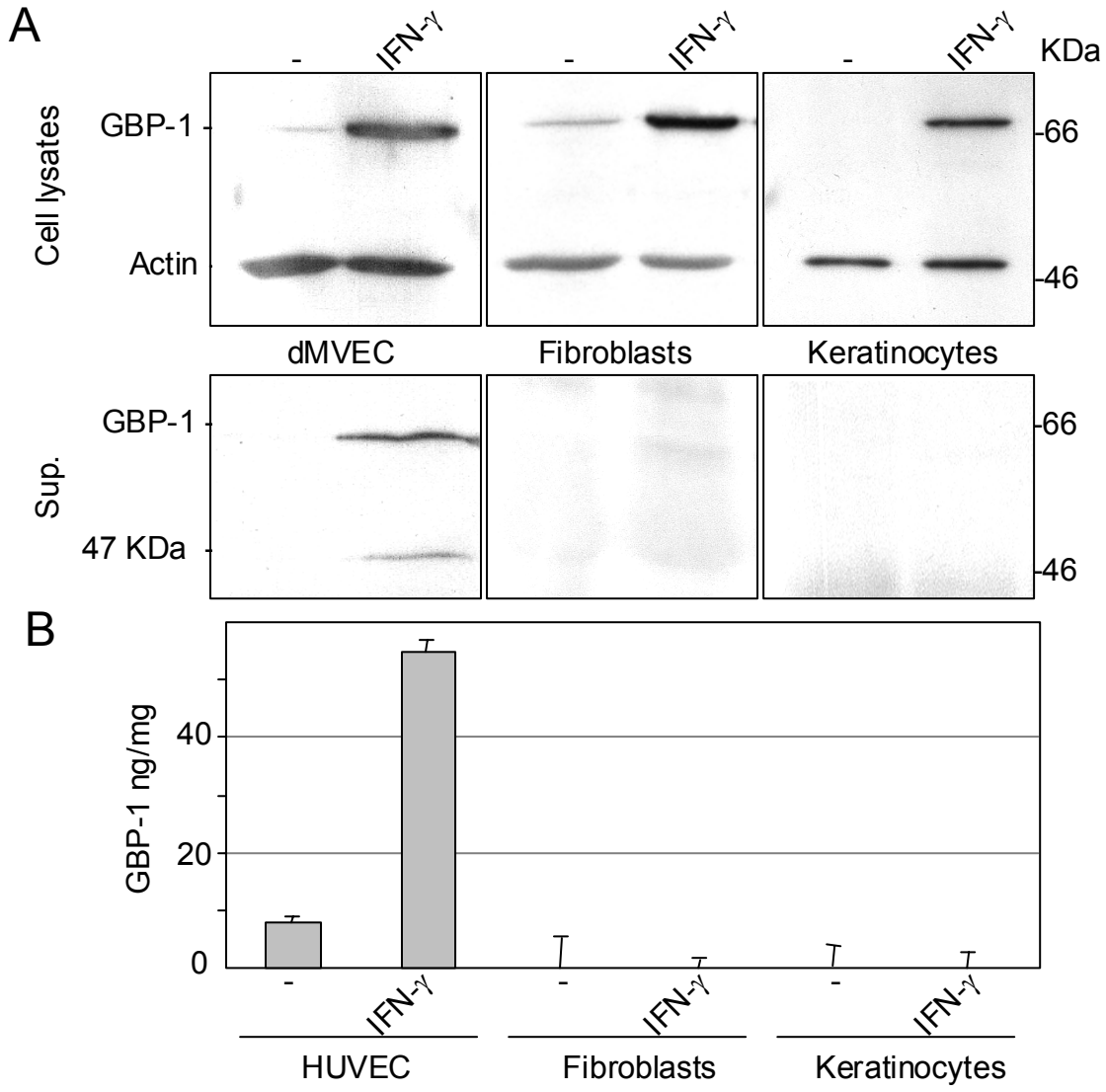


Figure 44: Investigation of the presence of GBP-1 in the cell culture supernatants of different cell types.
*(**A** and **B**) Primary EC (dMVEC and HUVEC), primary adult fibroblasts and the keratinocytic cell line Hacat were incubated with IFN-γ (100 U/ml) or with BSA (C) for 24 h. (**A,** upper panel) GBP-1 expression was analyzed in the cell lysates by Western blot using MAb 1B1. Actin staining shows that comparable amounts of protein extracts were loaded. (**A**, lower panel) GBP-1 was immunoprecipitated from the cell culture supernatants of the same cells (Sup) using a polyclonal anti-GBP-1 antibody followed by Western blot analysis using MAb 1B1. GBP-1 was detected in the supernatant of dMVEC only. In addition a band of about 47 kDa was detected. (**B**) The amount of secreted GBP-1 in the cell culture supernatant from HUVEC, fibroblasts and keratinocytes was measured by ELISA.*

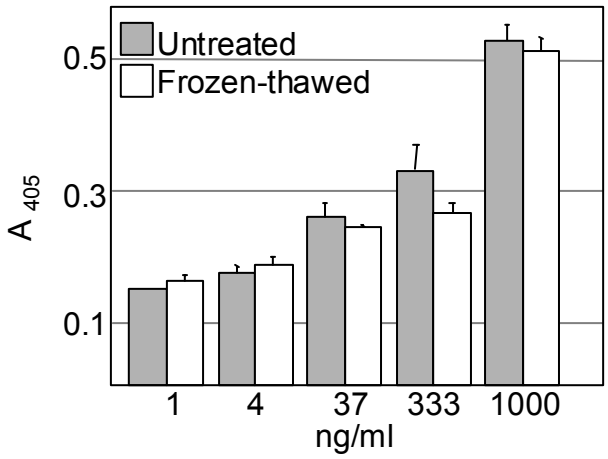


Figure 45: Effect of freeze-thawing on the detection of His-GBP-1 in the serum by ELISA. *Purified recombinant His-GBP-1 was gradually diluted in human serum (gray bars). The same solutions were frozen and thawed two times (white bars). GBP-1 concentrations were measured by ELISA. The absorbance was measured at 405 nm (A_{405}).*

4.5.1 Detection of GBP-1 in the plasma of patients under IFN-α treatment

IFN-α is often used in the clinical therapy of melanoma (Eggermont 2002). In addition, *in vitro* IFN-α has been shown to induce both GBP-1 expression and secretion (Figure 43). Therefore, the plasma of melanoma patients under IFN-α-therapy was regarded as an appropriate test system to determine whether circulating GBP-1 can be detected in the body fluids of patients with increased concentrations of IFN-α in the blood.

The amount of circulating GBP-1 was measured by ELISA in the plasma of three melanoma patients treated with IFN-α. Patients were treated with IFN-α ($20 \cdot 10^6$ U/m^2, intravenously) for four weeks (five days of treatment followed by two days of interruption). At day nine, the GBP-1 content in the plasma of the patients was 101 (± 3) ng/ml and 112 (± 11) ng/ml for two patients and was non detectable in one patient (Figure 46, white bars). At day twenty-eight after the beginning of the therapy GBP-1 concentrations in the plasma of the same patients increased to 168 (± 5) ng/ml, 248 (± 3) ng/ml and 165 (± 17) ng/ml, respectively (Figure 46, gray bars). These data indicated that in all patients IFN-α-therapy was accompanied by a significant increase of the content of soluble GBP-1 in the plasma (mean of the three patients: from 71.7 ± 60 ng/ml to 193.7 ± 47 ng/ml).

The detection of circulating GBP-1 at day twenty-eight of the therapy (the second day of interruption of IFN-α-treatment in the fourth cycle) indicated that circulating GBP-1 may be stable *in vivo*.

Altogether these results indicated, for the first time, that GBP-1 can be detected in the blood of patients that are exposed to increased concentrations of IC.

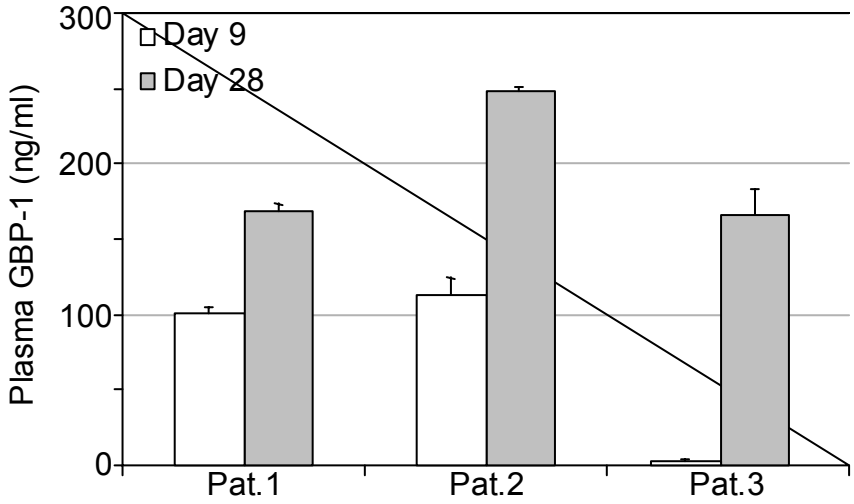


Figure 46: Amount of circulating GBP-1 protein in the plasma of IFN-α-treated melanoma patients*. Three melanoma patients were treated with IFN-α ($20 \cdot 10^6$ U/m^2, intravenously) for twenty-eight days (five days of treatment followed by two days of interruption). The amount of GBP-1 in the plasma at days nine (white bars) and twenty-eight (gray bars) of the therapy was measured by ELISA.*

4.5.2 Detection of GBP-1 in the plasma of AIDS patients

In AIDS patients IFN-γ, IL-1β and TNF-α are found in chronically elevated concentrations in the serum (Hober*, et al.* 1989; Emilie*, et al.* 1990; Vyakarnam*, et al.* 1991; Stürzl*, et al.* 1995; Ensoli*, et al.* 1998). In particular it has been shown that circulating IFN-γ is increased in early stage HIV-1 patients, but decreases in patients with full-blown AIDS (Ullum*, et al.* 1997; Twigg*, et al.* 1999; Huang*, et al.* 2000). Therefore, the plasma of HIV-1-infected patients was regarded as an appropriate test system to determine whether circulating GBP-1 can be detected in the plasma of patients with increased endogenous IFN-γ concentrations in the blood.

Thirty-eight HIV-1-infected patients were divided into groups following the definition of progression of the disease according to the U. S. Center for Disease Control (CDC) criteria for AIDS diagnosis. The groups were classified according the number of circulating CD4 positive cells (CD4+): group 1 = CD4+ cells > 500/mm^3 (n=8); group 2 = CD4+ cells 200-499/mm^3 (n=17) and group 3 = CD4+ cells < 200/mm^3 (n=13).

The content of soluble GBP-1 in the plasma of these patients was measured with the GBP-1 ELISA. A decrease of soluble GBP-1 concentration was observed in correlation with the

progression of the disease (median of GBP-1 concentrations. CDC1: 1836.3 ng/ml; CDC2: 475.1 ng/ml; CDC3: 199 ng/ml) (Figure 47, left panel). The correlation was significant (Pearson correlation coefficient, 1-tailed, $p < 0.01$).

The same patients were also grouped according to the CDC definition of progression of the disease. This definition is based on the appearance of the symptoms: group A = asymptomatic patients (n=18); group B = patients with some opportunistic infections (n=8) and group C = patients with full blown AIDS (n=4). For eight patients the symptoms at the time of the plasma extraction were not clear.

Also in this case a decrease of soluble GBP-1 concentration was observed in correlation with the progression of the disease (median of GBP-1 concentrations. CDC A: 982.0 ng/ml; CDC B: 188.9 ng/ml; CDC C:, 199.5 ng/ml) (Figure 47, right panel). Also in this case the correlation between decreasing GBP-1 concentrations and progression of the diseases was significant (Pearson correlation coefficient, 1-tailed, $p < 0.01$).

These results demonstrated that GBP-1 serum concentrations are inversely correlated with disease progression in AIDS patients.

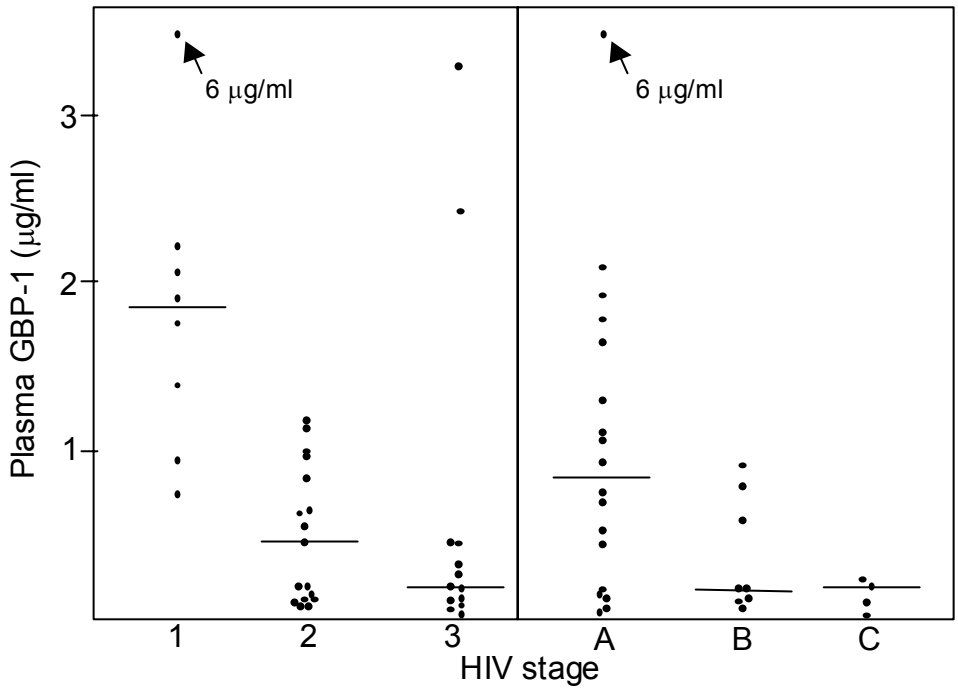


Figure 47: GBP-1 concentrations in the plasma of HIV-1-infected patients. *HIV-infected patients were divided into groups according to the CDC definition of progression of the disease. Group 1 = CD4+ cells > 500/mm^3 (n=8). Group 2 = CD4+ cells 200-499/mm^3 (n=17). Group 3 = CD4+ cells < 200/mm^3 (n=13). Group A = asymptomatic patients (n=18). Group B = patients with some opportunistic infections (n=8). Group C = patients with full blown AIDS (n=4). GBP-1 concentrations in the plasma of the patients were measured by ELISA. The two highest values are out of scale. For each group of patients the median is indicated by a horizontal line. Decreases of soluble GBP-1 concentrations correlated significantly with the progression of the disease according to CDC criteria for the number of CD4+ cells (left panel) and to CDC criteria for the phenotype of the disease (right panel) (in both cases Pearson correlation coefficient, 1-tailed, p<0.01).*

4.5.3 Detection of GBP-1 in the serum of patients with of inflammatory skin diseases

GBP-1 has been shown to be expressed in the blood vessels in the skin of patients affected by inflammatory skin diseases, but not in healthy skin (see Figure 28). In order to investigate whether GBP-1 may also be a serological marker of inflammation, GBP-1 concentration was determined in the serum of patients affected with different inflammatory skin diseases, including adverse drug reactions of the skin (n=16), urticaria (n=4), atopic dermatitis (n=12) and erythema exudativum multiforme (e. m.) (n=3). All these diseases are characterized by a local inflammatory response involving infiltration into the tissue of inflammatory cells and a systemic increase of IC, in particular of IFN-γ (Kapp 1993; Chodorowska 1998; Ackermann*, et al.* 1999; Livni*, et al.* 1999; Yawalkar*, et al.* 2000; Kaminishi*, et al.* 2002). As a control GBP-1 content in the sera of a group of 11 healthy donors was investigated.

The amount of circulating GBP-1 in the sera of these patients was measured by ELISA. The number of patients affected by each disease was limited. Therefore no reliable statement about the statistical distribution of the data could be done. As a consequence, the data were analyzed twice, assuming normal distribution (mean, T-test) as well as non-normal distribution (median, Mann-Whitney Test) of the data.

In none of the sera (100 %) of healthy donors GBP-1 could be detected (Figure 48, healthy, red circles). In contrast in the diseased group, twenty serum samples contained detectable GBP-1 concentrations (20/35 = 57 %) (Figure 48, diseased, black circles). GBP-1 concentrations in the serum of patients with inflammatory skin diseases (mean 163.1 ± 13 ng/ml, median 13.3 ng/ml) were significant higher (Mann-Whitney test, $p < 0.004$, one-tailed; T-test $p < 0.004$, two-tailed) as compared to the healthy control group assuming an average and median of 4 ng/ml (4 ng/ml is the smallest concentration of recombinant GBP-1 that could be detected in sera, see Figure 45) (Figure 48).

In a final step GBP-1 serum concentrations of each disease group were compared to the values obtained from the healthy persons. In almost all cases GBP-1 concentrations were significantly increased in the serum of diseased patients as compared to the healthy persons, no matter of a normal or non normal distribution of the data was assumed (Figure 49).

These data indicate that increased GBP-1 concentrations in the blood may be used as a marker of inflammatory disease in patients.

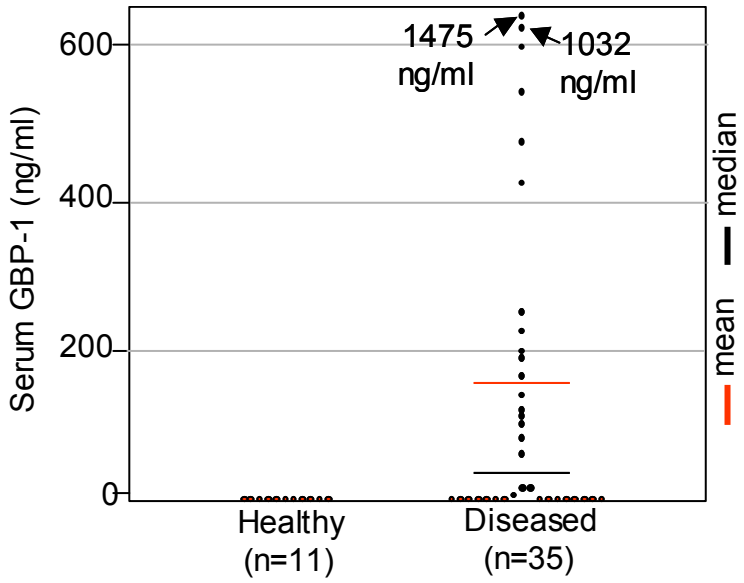
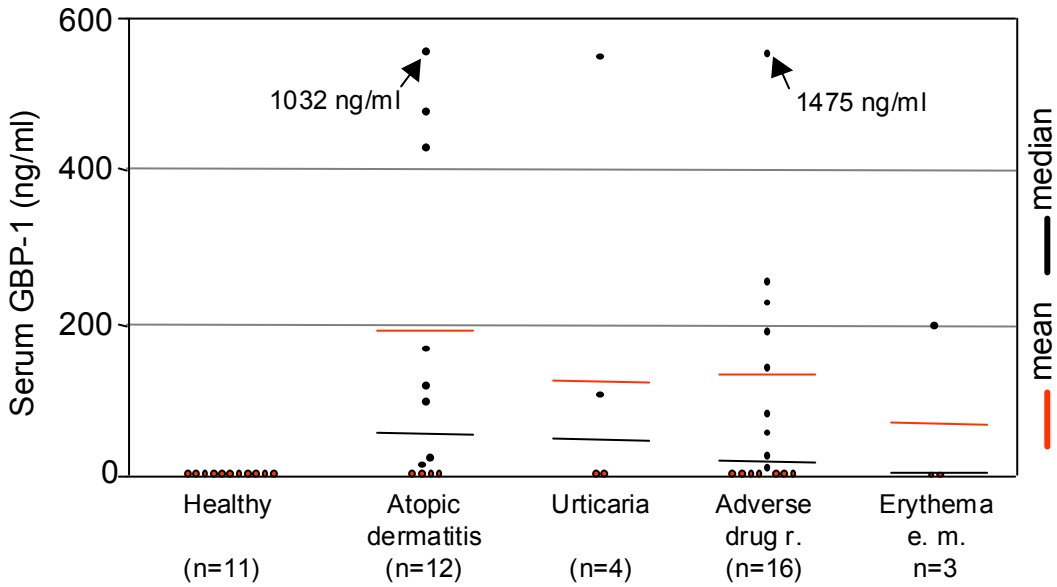


Figure 48: Amount of circulating GBP-1 in the serum of patient with inflammatory skin diseases. *Thirty-five patients affected by skin diseases with an inflammatory component including atopic dermatitis, urticaria, adverse reactions of the skin and erythema exudative multiforme were included in the diseased group (Diseased). The control group was composed of 11 healthy people (Healthy). The concentrations of GBP-1 in the serum were measured by ELISA. Red circles: undetectable GBP-1 concentrations. Statistical analysis was performed both assuming or non assuming a normal distribution. GBP-1 concentrations in "Diseased": mean 163.1 ± 13 ng/ml (red line), median 13.3 ng/m (black line). The two highest values are put of scale. The difference was significant. Mann-Whitney test, p < 0.004, one-tailed and T-test p < 0.004.*



GBP-1, Me. (ng/ml)	*N.d.*	189.4**	142.4**	155.7**	68.0**
GBP-1, Mn. (ng/ml)	*N.d.*	52.5**	42.7*	15.0*	2.0

Figure 49: Detection of circulating GBP-1 in the serum of patient with inflammatory skin diseases. *The same 35 patients and 11 healthy persons as in figure 48 were analyzed in detail. The number of patients in each group was: atopic dermatitis (n = 12), urticaria (n = 4), adverse reactions of the skin (adverse drug r., n = 16) and erythema exudativum multiforme (n = 3). The healthy control group contained 11 persons. Red circles: undetectable GBP-1 concentrations. Statistical analysis was performed both assuming or non assuming a normal distribution of the data. The concentrations of GBP-1 (mean, red line; median, black line) in the serum were measured by ELISA. N.d. = non detectable. Me. = mean, significance according to the T-Test: ** p<0.01, * p<0.05. Mn. = median, significance according to one-tailed Mann-Whitney Test: ** p<0.01, * p<0.05*

DISCUSSION

The main goal of this work was to investigate whether GBP-1 may be a novel marker to detect IC activation of endothelial cells (EC) *in vitro* and *in vivo*, both at the tissue level and in serological studies. In this framework, anti-GBP-1 antibodies were generated. The soluble recombinant His-GBP-1 used for the immunization of rats and rabbits was purified under native conditions, using a one step purification protocol. One of the monoclonal antibodies (MAbs) generated, named MAb 1B1, was specific for GBP-1 and did not recognize GBP-2 in Western blot analysis. Of note GBP-2 has 76 % homology to GBP-1 at the amino acid level (Table 1). In 2002 other GBP isoforms were described: GBP-3, GBP-4 and GBP-5 that share 22 %, 50 % or 65 % homology with GBP-1 respectively (Table 1). Therefore, it is likely that MAb 1B1 does not react with other GBP-1 isoforms and that it reacts specifically with GBP-1. MAb 1B1 was used for the detection of GBP-1 by immunohistochemistry in human tissue sections, which was one of the major goals of this work. Moreover, MAb 1B1 was employed for the development of a specific ELISA for the detection of GBP-1 in solution.

1 GBP-1 is a marker of the inflammatory cytokine-activated phenotype of endothelial cells *in vitro*

The gene encoding human GBP-1 was originally discovered among the major IFN-γ-induced genes (Cheng*, et al.* 1983; Cheng*, et al.* 1985; Decker*, et al.* 1989; Ruszczak*, et al.* 1990; Nantais*, et al.* 1996; Saunders*, et al.* 1999). In fact, GBP-1 mRNA expression has often been used as a marker to demonstrate IFN-γ-activation of cells in culture (Ucer*, et al.* 1986; van Loon*, et al.* 1991; Tnani*, et al.* 1999; Yang*, et al.* 1999; Kumar*, et al.* 2001). Moreover, during previous work in this laboratory it has been shown that not only IFN-γ, but also IL-1β and TNF-α induce GBP-1 mRNA expression in a dose-dependent manner. In particular, it has been shown that GBP-1 mediates the anti-proliferative effect of these IC (IFN-γ, IL-1β and TNF-α) on EC (see Introduction, paragraph **3.2**) (Guenzi*, et al.* 2001). In the present work the results obtained on GBP-1 at the mRNA level were confirmed at the protein level in EC.

EC express receptors for many different cytokines like IFNγ, IL-1α, IL-1β, TNF-α, IL-4, IL-6, IL-10, IL-18, C-C chemokines like MCP-1 and MIP-1β, C-X-C chemokines like PF4, IP-10, and SDF-1α and growth factors like bFGF, VEGF, Ang-2, PDGF B/B (Thornhill*, et al.* 1990a; Thornhill*, et al.* 1990b; Maruo*, et al.* 1992; Plate*, et al.* 1992; Maisonpierre*, et al.* 1997; Thommen*, et al.* 1997; Sanders*, et al.* 1998; Gentilini*, et al.* 1999; Murdoch*, et al.* 1999; Vasse*,*

et al. 1999; Salcedo*, et al.* 2000; Dzenko*, et al.* 2001; Madge*, et al.* 2001; Mallat*, et al.* 2001; Moore*, et al.* 2001). These receptors mediate the phenotypic changes of EC to the respective factors. Consequently, several cytokines, chemokines, and growth factors were tested for their ability to increase GBP-1 expression in HUVEC.

In agreement with the results obtained at the mRNA level, IFN-γ, IL-1β and TNF-α were found to increase GBP-1 expression in EC. In addition, also IL-1α and IFN-α increased GBP-1 expression in EC, but none of the other factors mentioned above.

Notably, all of the IC, which induced GBP-1 expression in EC, have been shown to inhibit proliferation of these cells (Frater-Schroder*, et al.* 1987; Friesel*, et al.* 1987; Schweigerer*, et al.* 1987a; Cozzolino*, et al.* 1990; Ruszczak*, et al.* 1990; Guenzi*, et al.* 2001). Other factors known to inhibit EC proliferation like IL-6, IL-10 and OSM, did not induce GBP-1 expression (May*, et al.* 1989; Takashima*, et al.* 1996; Moore*, et al.* 2001). This indicated that GBP-1 may characterize non-proliferating EC activated by IC, such as IFN-α, IFN-γ, IL-1α, IL-1β or TNF-α.

EC activation induces structural and functional alterations of the endothelium and plays a key role in angiogenesis and inflammation (Pober, et al. 1986; Cotran, et al. 1988; Pober 1988; Cotran, et al. 1990; Augustin, et al. 1994; Folkman 1995; Carmeliet*, et al.* 2000). In particular, EC activation in tumour angiogenesis (Nicosia*, et al.* 1983; Folkman*, et al.* 1991; Plate*, et al.* 1992; Kim*, et al.* 1993; Fan*, et al.* 1995; Folkman 1995; Siegel*, et al.* 1997; Desai*, et al.* 1999; Carmeliet*, et al.* 2000; St Croix*, et al.* 2000), atherosclerosis (Tan*, et al.* 1999; Baumgartl*, et al.* 2001; Roesen*, et al.* 2001; Cascieri 2002), adverse drug reaction of the skin (Yawalkar*, et al.* 2000), angioedema (Cotran*, et al.* 1990; Livni*, et al.* 1999) or endothelial-derived tumors like Kaposi's sarcoma (Ensoli*, et al.* 1994; Uccini*, et al.* 1994; Stürzl*, et al.* 1995; Ensoli*, et al.* 1998; Fiorelli*, et al.* 1998; Samaniego*, et al.* 1998; Sirianni*, et al.* 1998; Stürzl*, et al.* 1999; Ensoli*, et al.* 2000; Ensoli*, et al.* 2001; Stürzl*, et al.* 2001) represents a time- and dose-integrated response to various stimuli originating from the blood and/or from neighbouring cells and tissues. Investigation of the appearance of the different activation phenotypes of EC may help to elucidate the complex network of different cellular activations in these diseases.

In vivo, different factors can lead to activated EC. Some of these factors induce the same activity and can be put together in groups with functional homology. Two groups that play a major role in EC activation, are for example: (**i**) inducers of EC proliferation like VEGF and bFGF (AGF) (Montesano et al. 1986; Folkman, et al. 1987; Folkman and Klagsbrun 1987; Schweigerer et al. 1987; Leung et al. 1989; Keck et al. 1989; Wilting et al. 1993; Gospodarowicz 1991; Melder et

al. 1996), and (**ii**) inhibitors of EC proliferation and inducers of leukocyte adhesion like IFN-γ, IL-1β and TNF-α (IC) (Friesel, et al. 1987; Holzinger, et al. 1993; Jaramillo, et al. 1995; Neary, et al. 1996). Notably, IC and AGF compete in the induction of a proliferative (mediated by AGF, Figure 50, red squares) or a non-proliferative (mediated by IC, Figure 50, blue circles) phenotype of EC.

The suitability of GBP-1 as a marker of IC activation of EC at simultaneous presence of IC and AGF was investigated using two approaches:

(1) Pre-incubation of EC with AGF did not affect IC-induced GBP-1 expression in EC. This fact is an advantage for a molecular marker of IC-activated EC. EC that are exposed to IC can express GBP-1 independently of a previous stimulation.

(2) AGF applied simultaneously with IC inhibited IC-induced GBP-1 expression. This kind of regulation is also an advantage for a marker of IC-activated EC. In an environment were both AGF and IC are present, the level of GBP-1 may indicate the relative concentration of IC present in the tissue.

This demonstrated that GBP-1 may be a suitable marker of IC activation of EC in the presence of complex stimulations. In addition, the second point showed that the major activation pathways of EC, namely the inflammatory cytokines pathway and the angiogenic growth factor pathway converge in the regulation of GBP-1 expression.

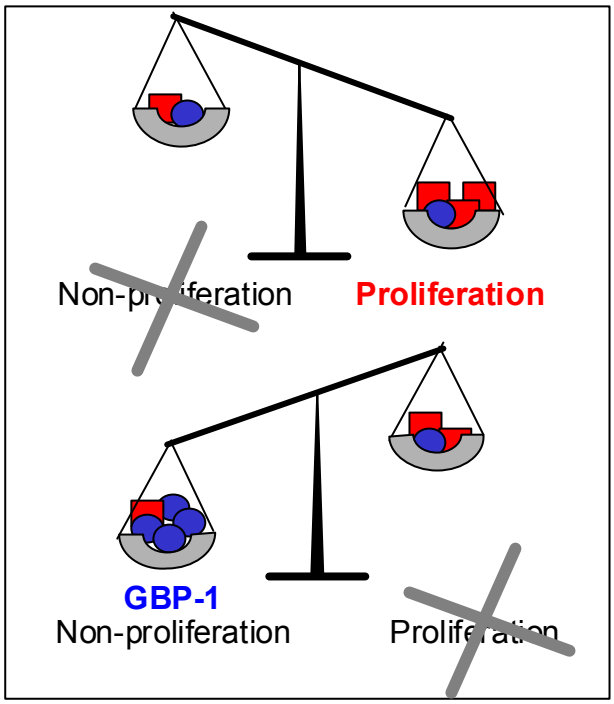


Figure 50: Model of GBP-1 as a marker to dissect the interplay of IC and AGF. *EC activation is mediated by a variety of soluble factors originating from the blood or from neighbouring cell. Two groups of factors are: IC (blue circles) that induce a non-proliferative, adhesion competent phenotype of EC and AGF (red squares) that activate EC proliferation. EC can switch between a proliferating and a non-proliferating phenotype. When AGF predominate, the IC-induced GBP-1 expression is inhibited, and EC proliferate. By contrast, if IC predominate, GBP-1 is expressed and EC are in a non-proliferating phenotype.*

In addition, repeated stimulation of EC with IL-1β did not result in adaptation but resulted in a constantly high expression of GBP-1. This demonstrated that GBP-1 may indicate also long-term activation of EC by IC. This further qualifies GBP-1 as a marker of IC-activated EC.
In this framework the use of GBP-1 as a marker will be useful to determine the temporal and spatial appearance of the IC-activated phenotype of EC in inflammatory processes and during angiogenesis. This will help to decipher the multicellular and multifactorial interactions regulating pathological changes of the endothelium and may provide a platform for the development of novel anti- and pro-angiogenic approaches targeting distinct activation phenotypes of EC.

2 Subcellular localization of GBP-1 in endothelial cells

GBP-1 localization studies in EC have shown that GBP-1 is a cytoplasmic protein. GBP-1 localization was the same irrespectively of the cytokine used for the induction of its expression. Moreover, GBP-1 partially localized in the endoplasmic reticulum (ER). The latter finding was shown by colocalization studies of GBP-1 and the ER marker concanavalin A. ER localization of GBP-1 is in agreement with the subcellular localization of other large GTPases (see Table 1). For example, the dynamin-like protein-1 DLP-1 has been shown to localize in the endoplasmic reticulum and the interferon-induced large GTPase MxA has been shown to localize in the smooth endoplasmic reticulum (Yoon*, et al.* 1998; Pitts*, et al.* 1999; Accola*, et al.* 2002)
GBP-1 carries a CAAX isoprenylation motif at its C-terminus (Asundi*, et al.* 1994; Nantais*, et al.* 1996). Isoprenylation is a post-transcriptional modification that can facilitate association of proteins with intracellular membranes. Carboxy methyltransferases (enzymes involved in the attachment of an isoprenoid residue to the CAAX isoprenylation motifs) have been shown to localize in the ER both in *Saccharomyces cerevisiae* and in mammals (Dai*, et al.* 1998; Schmidt*, et al.* 1998; Choy*, et al.* 1999). Notably, GBP-1 isoprenylation is compatible with the finding that a significant fraction of cellular GBP-1 protein is present in the ER.
In addition to ER localization, stainings for intracellular GBP-1 revealed a granular pattern. This result is well in agreement with the observation of a granular appearance of other large GTPases including DLP-1 and GBP-2 (Yoon*, et al.* 1998; Pitts*, et al.* 1999; Vestal*, et al.* 2000). Notably, the granular pattern of these large GTPases has been attributed to their localization in cytoplasmic vesicles (Vestal*, et al.* 2000; Danino*, et al.* 2001). However, the kind of vesicles containing GBP-2 could not be defined as yet (Vestal*, et al.* 2000).

GBP-1 did not localize in known organelles like caveolae or lysosomes. As mentioned in the introduction, intracellular transport vesicles in eukaryotic cells are coated with proteins [reviewed in (Kirchhausen 2000), see also (Schekman 1998; Schekman 2002)]. These proteins, together with sphingolipid-cholesterol rafts make the membranes of these vesicles insoluble in the detergent Triton-X100 at 4 °C (Schekman, et al. 1996; Simons, et al. 1997; Helms, et al. 1998; Schekman 1998; Schekman 2002). It has been shown that acylated proteins are associated with detergent-insoluble membrane fractions, for example Rap1 that is a monomeric GTPase that is closely related to Ras (Melkonian, et al. 1999). By contrast, prenylated proteins are largely excluded from detergent-resistant membrane fractions; however, some prenylated proteins have also been found in such detergent-insoluble fractions (Melkonian, et al. 1999). GBP-1 prenylation could target GBP-1 to coated vesicles containing detergent-insoluble membranes and cause the granular pattern observed in GBP-1 subcellular localization. Therefore, the detergent solubility of GBP-1 was investigated. Detergent extraction experiments showed that about 16.7 % of intracellular GBP-1 could be recovered in detergent-insoluble fraction. This result is in agreement with observations on GBP-1 isoprenylation in the promyelocytic cell line HL-60. In HL-60 cells GBP-1 has been shown to be isoprenylated, but only 15 % of isoprenylated GBP-1 was found to be membrane-associated (Nantais*, et al.* 1996). The latter finding was demonstrated by subcellular fractionating (Nantais*, et al.* 1996). Therefore, the granular pattern of GBP-1 staining may be due to GBP-1 localization in detergent-insoluble vesicles.

In conclusion, it has been shown that GBP-1 is a cytoplasmic protein that partially localizes in the ER. A fraction of GBP-1 is localized in granular structures and may be membrane-associated, possibly due to isoprenylation.

3 GBP-1 is a marker of non-proliferating, inflammatory cytokine-activated endothelial cells *in vivo*

A detailed examination of GBP-1 expression in various human tissues demonstrated that GBP-1, in contrast to the *in vitro* situation, is highly associated with vascular EC. Besides EC, GBP-1 expression *in vivo* was detected in some mononuclear cells. The latter findings may be explained with the observation that monocytes *in vitro* are expressing high basal levels of GBP-1. The EC-association of GBP-1 was confirmed by double labelling studies for simultaneous detection of the endothelial cell-associated antigen CD31 and GBP-1 (Figure 32).

Different control stainings were performed in order to show the specificity of GBP-1 staining. First, the staining procedure was carried out without the primary antibody (rat MAb 1B1), no signals were obtained indicating that the secondary antibody did not bind unspecifically to the tissue sections. Second, staining was performed with a rat isotype control antibody. This indicated that the constant chain of the rat antibody did not bind unspecifically to the tissue sections. Third, the primary antibody was pre-adsorbed with an excess of purified recombinant GBP-1-His protein. Also in this case no signal could be detected, indicating that the antibody did not bind to other proteins when the GBP-1 binding sites were selectively blocked.

Altogether these results showed that GBP-1 is highly associated with EC *in vivo* and suggested that the regulation of GBP-1 expression *in vivo* is more strictly regulated than *in vitro* and that GBP-1 may play an important role in the activation of the endothelium *in vivo*.

Notably, EC in healthy skin did not express GBP-1. This was exploited in order to investigate whether GBP-1 expression in EC *in vivo* may be also induced by IC. GBP-1 expression was investigated in three different vascularized and IC-regulated diseases of the skin (see Introduction, paragraph **2.5**), namely KS, psoriasis and adverse drug reactions of the skin (Gomi*, et al.* 1991; Kapp 1993; Chodorowska 1998; Ackermann*, et al.* 1999; Yawalkar*, et al.* 2000; Stürzl*, et al.* 2001). The skin was chosen because inflammatory processes are clinically most apparent and most easily accessible when they occur in the skin. The skin has a rich blood supply and inflammatory diseases involving the skin commonly include inflammatory activation of the underlying blood vessels.

In contrast to healthy skin, GBP-1 was detected in each of the three inflammatory diseases of the skin. GBP-1 was selectively expressed in vascular EC. These findings, together with the described locally restricted expression of IC in these diseases (Gottlieb*, et al.* 1988; Kapp 1993; Stürzl*, et al.* 1995; Fiorelli*, et al.* 1998; Ackermann*, et al.* 1999; Hari*, et al.* 1999; Ensoli*, et al.* 2000; Guenzi*, et al.* 2001; Stürzl*, et al.* 2001), indicated that IC may upregulate GBP-1 expression. This was further confirmed by a detailed analysis of GBP-1 expression in KS. As mentioned above KS is an angio-proliferative disease that initiates in the course of a reactive process driven by the same IC which have been shown to induce GBP-1 expression (Ensoli*, et al.* 2000; Stürzl*, et al.* 2001). Expression of all these cytokines has been demonstrated in KS tissue sections (Gottlieb*, et al.* 1988; Kapp 1993; Stürzl*, et al.* 1995; Fiorelli*, et al.* 1998; Ackermann*, et al.* 1999; Hari*, et al.* 1999; Guenzi*, et al.* 2001;). The main source of IC in KS lesion are infiltrated monocytes (Stürzl*, et al.* 1995; Fiorelli*, et al.* 1998). Immunohistochemical staining for GBP-1 and monocytes using the monocytic-marker CD68

showed that GBP-1 was selectively expressed in vessels that were surrounded by numerous perivascular CD68-positive monocytes. This suggested that in KS lesions the IC released by infiltrated monocytes may induce GBP-1 expression.
In addition, GBP-1 expression was analyzed in KS tissue sections in which early and late developmental stages of KS were present simultaneously. Simultaneous detection of GBP-1 and CD31 indicated that in these sections GBP-1 was predominantly expressed in the areas presenting an early stage histology. This is in agreement with the higher IC expression in these early KS stages (Brooks 1986; Stürzl*, et al.* 1995; Ensoli*, et al.* 2000; Stürzl*, et al.* 2001). By contrast, in areas with nodular late stage histology, GBP-1 expression was clearly lower, which is in agreement with the increased expression of AGF which has been reported in late stage KS lesion (Xerri*, et al.* 1991; Ensoli*, et al.* 1994; Cornali*, et al.* 1996;).

As discussed so far, GBP-1 is a marker of IC activation of EC both *in vitro* and *in vivo*. IC are known to inhibit EC proliferation (Friesel*, et al.* 1987; Holzinger*, et al.* 1993; Jaramillo*, et al.* 1995; Neary*, et al.* 1996). In order to investigate whether GBP-1 may characterize IC-activated non-proliferating EC, double and triple labeling studies for simultaneous detection of GBP-1, of the proliferation marker Ki67, and the endothelial cell-associated antigen CD31 were performed in KS lesions. These immunohistochemical studies showed that GBP-1 expression is only expressed in non-proliferating vessel EC (CD31-positive, Ki67-negative). By contrast, GBP-1 was never detected in proliferating EC (CD31-positive, Ki67-positive). GBP-1 expressing vessels were surrounded by numerous infiltrating monocytes. Monocytes produce IC that inhibit EC proliferation (Fiorelli*, et al.* 1998). In contrast, bFGF and VEGF are released from the KS spindle cells that are predominantly present in late stage KS lesions and activate EC proliferation (Xerri*, et al.* 1991; Ensoli*, et al.* 1994; Cornali*, et al.* 1996).
In summary, these data indicated that GBP-1 is selectively upregulated by IC in inflammatory skin diseases and characterizes the IC-activated non-proliferating phenotype of EC *in vivo*.

4 GBP-1 is a secreted protein

GBP-1 expression in tissue sections was highly associated with EC and indicated IC activation of these cells. In addition, in EC GBP-1 was localized in granular structures, which indicated that this protein may be targeted to a secretory pathway and released from the cells. Secretion of proteins is important because it provides a mean by which EC can rapidly and selectively alter the microenviroment of an individual vascular bed (Datta*, et al.* 2001, see also

Introduction, paragraph **1**). Secreted GBP-1 may be a marker of inflammatory activation of blood vessels that may be easily accessible and detectable by serological methods.

GBP-1 was secreted by both micro- and macro-vascular EC under IFN-γ and IFN-α stimulation as assessed both by immunoprecipitation and ELISA. Notably, GBP-1 was not secreted by fibroblasts nor by keratinocytes. This indicated that GBP-1 secretion may be EC-specific.

Additional experiments with transduced HUVEC that expressed GBP-1 constitutively, showed that GBP-1 is also secreted into the cell culture supernatant by these cells. This suggested that the mechanism of GBP-1 secretion is independent from IFN-γ or IFN-α stimulation. Of note, a GBP-1 mutant with a deletion of the CAAX isoprenylation was not secreted by these cells. This indicated that isoprenylation may be necessary for GBP-1 secretion. The latter observation is in analogy with the secretion of the a-factor (one mating pheromone of *Saccharomyces cerevisiae*) that is released *via* the multidrug resistance (MDR) pathway. Also the a-factor, has to be isoprenylated before secretion (Kuchler*, et al.* 1989; McGrath*, et al.* 1989; Michaelis 1993; Caldwell*, et al.* 1994).

GBP-1 secretion occurred independently of cell death as indicated by the following points:

(1) No increase of the abundant cytosolic proteins GAPDH or LDH could be detected in the cell culture supernatants of EC after IFN-γ stimulation. This was assessed by immunoprecipitation of GAPDH and by the LDH activity assay respectively. Measurement of LDH activity is considered as the most sensitive approach to detect alterations in cell permeability and non-specific release of intracellular proteins (Rubartelli*, et al.* 1990; Ensoli*, et al.* 1993; Chang*, et al.* 1997).

(2) The amount of secreted GBP-1 was almost 10 % of total intracellular GBP-1. By contrast, the number of cells with decreased membrane permeability was only 1 % of the total cell number. The number of cells with decreased membrane permeability is too small to explain the high concentration of GBP-1 in the cell culture supernatants of IFN-γ-treated HUVEC.

(3) In primary adult fibroblasts no GBP-1 could be detected in the cell culture supernatant. Primary fibroblasts are sensitive to cell culture conditions. After IFN-γ stimulation primary fibroblasts expressed comparable amount of GBP-1 as HUVEC. If GBP-1 in the cell culture supernatant was due to cell death, it should be detectable also in cell culture supernatants of IFN-γ-treated fibroblasts. However, this was not the case.

(4) GBP-1 secretion could be inhibited by incubation of the cells at room temperature, this indicated that GBP-1 secretion is an energy-dependent process. In addition, like described in point (3), if GBP-1 in the cell culture supernatant was due to dead cells, it

should be detectable also in cell culture supernatants of IFN-γ-treated HUVEC when they are incubated at room temperature. This was not the case, and further supported that GBP-1 is actively secreted.

Proteins that are secreted through the classical secretion pathway are targeted to the endoplasmic reticulum by a leader peptide signal [reviewed in (Harter, *et al.* 2000; Allan, *et al.* 2002; Joiner, *et al.* 2002)]. Afterwards vesicles are budding from the endoplasmic reticulum and transport the respective proteins to the Golgi apparatus (Schekman, *et al.* 1996; Schekman 2002). Finally, secretory vesicles bud from the Golgi apparatus and fuse with the plasma membrane and reverse the included proteins into the cell culture supernatant (Helms, *et al.* 1998; Schekman 1998; Kirchhausen 2000). Fusion of vesicles from each compartment to another involves soluble NSF attachment protein (SNAP) receptors machinery (SNAREs) and ADP-ribosylation factors (ARFs) guanine nucleotide-exchange factors (Schekman, *et al.* 1996; Helms, *et al.* 1998; Schekman 1998; Chardin, *et al.* 1999; Niles, *et al.* 1999; Kirchhausen 2000; Yamaji, *et al.* 2000; Schekman 2002). Most SNAREs are C-terminally anchored integral membrane proteins capable of entering into an interaction with other SNARE proteins (Tooze, *et al.* 2001; Schekman 2002).

In order to investigate whether GBP-1 may be secreted through the classical secretion pathway, first a computer-assisted sequence analysis of GBP-1 was performed. No canonical leader signal peptide sequence could be identified. Several physiologically important proteins also lack a classical signal sequence. For example the angiogenic factors aFGF and bFGF (Prudovsky, *et al.* 2002) or IL-1β (Rubartelli, et al. 1990). Second, modulation of GBP-1 secretion was performed using different pharmacological agents. These agents are commonly employed for the inhibition of the classical or for alternative secretion pathways.

For example, monensin and brefeldin A (BFA) are inhibitors of the classical secretion pathway (Rubartelli, *et al.* 1990; Jackson, *et al.* 1995; Chang, *et al.* 1997; Soderberg, *et al.* 2000; Taraboletti, *et al.* 2000).

Monensin binds Na+, K+ and protons, causing hyper-polarisation of the plasma membrane without affecting the intracellular pH, ATP pool, nor protein synthesis. Monensin causes accumulation of proteins in the Golgi apparatus and a delayed secretion of unprocessed proteins. (Table 5)**.** Monensin increased GBP-1 secretion. It has been shown that monensin can increase the secretion of proteins that do not follow the classical secretion pathway such as IL-1β (Rubartelli *et al.* 1990) (Table 5). This indicated that GBP-1 may not be secreted by the classical secretion pathway.

BFA causes a reversible redistribution of Golgi stacks to the ER (Misumi*, et al.* 1986). Moreover, it has been shown that BFA blocks ARF-GDP complexes, creating an abortive complex that sequestrates exchange factors necessary for intracellular vesicle fusion processes (Table 5) (Sata*, et al.* 1999). BFA inhibited GBP-1 secretion. However, this effect was not due to an effect on GBP-1 secretion but was due on an inhibition of intracellular GBP-1 expression. In order to explain the effect of BFA on intracellular GBP-1, a computer-assisted sequence analysis was performed. BFA is known to bind ARF-GDP-complexes (Sata*, et al.* 1999; Yamaji*, et al.* 2000). Mammalians contain three classes of ARF_s with different sensitivities to BFA [reviewed in (Chardin*, et al.* 1999; Yamaji*, et al.* 2000)]. All known ARF_s contain a Sec7 domain with a consensus motive that binds BFA in its GTP-binding domain (Sata*, et al.* 1999). The consensus motive in Sec7 has the sequence Asp-X-X-X-X-Gln-X-X-X-X-Met, where X can be any amino-acid. In the nucleotide binding domain of GBP-1 (cap domain) a partially similar sequence can be found: $\underline{\text{Asp}}^{262}$-X-X-X-X-$\underline{\text{Gln}}$-$\underline{\text{Gln}}$-X-X-X-X-$\underline{\text{Cys}}^{273}$ (Prakash*, et al.* 2000a). Of note, Cys like Met is a sulphur-containing amino. Therefore, BFA might form a complex with GBP-1 leading to its intracellular degradation. Of note, this motif was not found in other members of the large GTPases family like GBP-2, dynamin or MxA.

Methylamine and verapamil are modulators of non classical secretion pathways. Both substances have been shown to increase secretion of aFGF (Table 5). aFGF is released form the cells through a non-classical secretion pathway with a poorly characterized mechanism (Prudovsky*, et al.* 2002). Methylamine and verapamil also increased GBP-1 secretion. This suggested that the secretory pathway of GBP-1 my reveal similar with that of aFGF.

Moreover, decreasing serum concentrations had no effect on GBP-1 secretion although they have been shown to affect the secretion of proteins including the secretion of aFGF and IL-1β (Jackson*, et al.* 1995; Rubartelli*, et al.* 1990). Therefore, the regulation of GBP-1 secretion reveal also distinct differences from that of aFGF.

These results, together with the analogy to the a-factor [the mating pheromone of *Saccharomyces cerevisiae* which has an isoprenylation-dependent secretion *via* the MDR pathway (Caldwell*, et al.* 1994)] suggested that GBP-1 secretion does not follow the classical secretion pathway. Instead, the mechanism of GBP-1 secretion may have some similarities with the ones of IL-1β and aFGF that both are secreted *via* alternative pathways, independent of a leader peptide signal (compare IL-1β, aFGF and GBP-1, Table 5).

Table 5: Effects of some pharmacological agents and non standard incubation conditions on the secretion of different proteins.

Treatment	Protein	Effect	Cells	References	Mechanism
BrefeldinA (Inhibition of the classical secretion pathway)	MMP-2	↓	HUVEC	Taraboletti, *et al.* 2000	Redistribution of Golgi stacks to ER. Reversible effect. Blocks ARF-GDP complexes, creating an abortive complex that sequestrates exchange factors necessary for intracellular vesicle fusion processes.
	ATP (HST)	—	BAEC	Hisadome, *et al.* 2002	
	aFGF	↑	NIH 3T3	Jackson, *et al.* 1995	
	Thio. reductase	↓	Monocytes	Soderberg, *et al.* 2000	
	IL-1β	↑	Monocytes	Rubartelli, *et al.* 1990	
	Albumin	↓	Rat hepat.	Misumi, *et al.* 1986	
	Haptoglobin	↓	Rat hepat.	Misumi, *et al.* 1986	
	Tat	—	COS-1	Chang, *et al.* 1997	
	GBP-1	↓(i)	HUVEC		
Monensin (Inhibition of the classical secretion pathway)	MMP-2	↓	HUVEC	Taraboletti, *et al.* 2000	Binds Na+, K+ and protons, causing hyper-polarisation of the PM without affecting intracellular pH, ATP pool, nor protein synthesis. Causes accumulation of proteins in the Golgi and delayed secretion of unprocessed proteins.
	IL-1β	↑	Monocytes	Rubartelli, *et al.* 1990	
	Thio. reductase	↓	Monocytes	Soderberg, *et al.* 2000	
	aFGF	—(↑)	NIH 3T3	Jackson, *et al.* 1995	
	Tat	—	COS-1	Chang, *et al.* 1997	
	GBP-1	↑	HUVEC		
Methylamine	macroglobulin	↓(c)	Swiss 3T3-4	Maxfield 79	Inhibition of non classical secretion pathway.
	aFGF	↑	NIH 3T3	Jackson, *et al.* 1995	
	IL-1β	↓	Monocytes	Rubartelli, *et al.* 1990	
	GBP-1	↑	HUVEC		
Verapamil	ATP (HST)	↓	BAEC	Hisadome, *et al.* 2002	Inhibits volume regulated anion channels and the multidrug resistance pathway.
	aFGF	↑	NIH 3T3	Jackson, *et al.* 1995	
	GBP-1	↑	HUVEC		
Decreased incubation temperature	Tat	↓	COS-1	Chang, *et al.* 1997	Slows down the cellular metabolism and reduces intracellular ATP pool.
	IL-1β	↓	Monocytes	Rubartelli, *et al.* 1990	
	aFGF	↓	NIH 3T3	Jackson, *et al.* 1995	
	GBP-1	↓	HUVEC		
Decreased serum concentra-tion	Tat	↑	COS-1	Chang, *et al.* 1997	Different undefined components like growth factors influence various cellular functions.
	IL-1β	↓	Monocytes	Rubartelli, *et al.* 1990	
	aFGF	↓	NIH 3T3	Jackson, *et al.* 1995	
	GBP-1	—	HUVEC		

MMP-2 = matrix metallo-proteinase 2; HST = heat-shock induced; Thio. = thioredoxin; i = intracellular; c = clustering. Hepat. = hepatocytes. PM = plasma membrane. ↓ = inhibition. ↑ = increase; — = no effect.

Interestingly, a smaller protein (≅ 47 kDa) which reacted with MAb 1B1 was detected only in the cell culture supernatants of the cells, but not in the cell lysates. This fragment may be a processed form of GBP-1. Other proteins that are secreted without a signal peptide undergo also processing prior to secretion, for example IL-1α and IL-1β, thioredoxin reductase or albumin (Misumi, *et al.* 1986; Rubartelli, *et al.* 1990; Soderberg, *et al.* 2000). Notably, proteins exist in which the processed mature form and the precursor form have different biological activities like for IL-1α, where only the precursor is able to regulate EC migration (McMahon, *et al.* 1997). Thus both secreted GBP-1 and the 47 kDa fragment should be considered for further investigations of the biologically activity of extracellular GBP-1.

In summary, GBP-1 has been found to be a secreted large GTPase. GBP-1 likely does not follow the classical secretion pathway. GBP-1 secretion pathway reveals some similarities with the secretion pathways followed by aFGF and IL-1β.

5 GBP-1 in the serum as a serologically accessible marker of IC-activation of endothelial cells

GBP-1 expression has been shown to be upregulated in patients with inflammatory skin diseases *in vivo* and to be secreted by EC upon IFNs stimulation in physiological concentrations *in vitro* (Lubeseder-Martellato*, et al.* 2002). Therefore, circulating GBP-1 in the blood may be an easily accessible marker to detect inflammatory activation of blood vessels in patients. In addition, *in vitro* secreted GBP-1 could be detected after 48 h from the IFNs induction and GBP-1 was stable in frozen serum samples. These facts, together with the availability of the developed GBP-1-ELISA, the limited amount of sample needed to detected secreted GBP-1 enables and facilitates the screening of archived blood samples.

In order to investigate this, in a first approach GBP-1 concentrations were determined in the plasma of patients under IFN-α-therapy. IFN-α-therapy is often used in the clinical treatment of melanoma (Eggermont 2002). The respective patients were treated with IFN-α ($20 \cdot 10^6$ U/m^2, intravenously) for four weeks (five days of treatment followed by two days of interruption). Continuosly increased systemic IFN-α levels may induce GBP-1 expression in EC and subsequent release. In fact, a significant increase of circulating GBP-1 concentration was observed from day nine to day twenty-eight of the IFN-α-therapy of the patients (mean of three patients: from 71.7 ± 60 ng/ml to 193.7 ± 47 ng/ml). Notably, GBP-1 could be detected in the plasma of these patients at day nine after begin of the therapy (this corresponds to the second day after a new intravenous IFN-α cycle). In addition, a constant increase of circulating GBP-1 could be detected at day twenty-eight of the therapy (the second day of interruption of intravenous IFN-α in the fourth cycle). The latter indicated that circulating GBP-1 may be stable in the blood of these patients. Altogether these results showed that GBP-1 is present in increased concentrations in the blood of patients treated with IFN-α. Therefore, soluble GBP-1 may be a marker of IC activation of EC *in vivo*.

In a next step, GBP-1 concentrations were determined in the serum of patients with inflammatory skin diseases. The group of patients was suffering of adverse reaction of the skin, urticaria, atopic dermatitis and erythema. These disease are all characterized by a local

inflammatory reaction (Livni*, et al.* 1999; Yawalkar*, et al.* 2000; Kaminishi*, et al.* 2002). In the patient groups a significant increase of GBP-1 in the serum was observed (adverse drug reaction of the skin, 155.7 ng/ml; urticaria, 142.4 ng/ml; atopic dermatitis, 189.4 ng/ml; erythema, 68 ng/ml) as compared to healthy control group (undetectable GBP-1 amounts). This indicated that GBP-1 is secreted *in vivo* during inflammatory conditions and that it can be detected in the blood, although the inflammation was locally restricted.

Finally, GBP-1 concentrations were determined in the plasma of HIV-infected patients. It has been shown that HIV-infected patients have increased serum levels of IC that in turn activate the endothelium (Hober*, et al.* 1989; Emilie*, et al.* 1990; Vyakarnam*, et al.* 1991; Stürzl*, et al.* 1995; Zietz*, et al.* 1996; Ensoli*, et al.* 1998; Ensoli*, et al.* 2000; Stürzl*, et al.* 2001). Notably, the amount of circulating GBP-1 decreased with the progression of the disease in these patients (median: CDC1, 1836.3 ng/ml; CDC2, 475.1 ng/ml; CDC3, 199 ng/ml). It is also has been reported that IFN-γ concentrations are increased in early stage HIV-1 patients, but decreased in patients with full-blown AIDS (Ullum*, et al.* 1997; Twigg, et al. 1999; Huang, et al. 2000). Because chronic inflammatory activation of the blood vessels endothelium has been shown to occur in HIV-infected patients, circulating GBP-1 in HIV-infected patients may be released by activated EC (Zietz*, et al.* 1996).

Altogether these preliminary clinical data indicate that circulating GBP-1 can be detected *in vivo* in a wide variety of inflammatory conditions, for example GBP-1 can be detected in the serum of patients affected by inflammatory skin diseases. The concentrations of circulating GBP-1 were increased from day 9 to day 28 in melanoma patients under IFN-α-therapy. Circulating GBP-1 concentrations decreased in HIV-1 affected patients in correlation with the progression of the disease and the reported decrease of circulating IFN-γ concentrations in these patients. Therefore, circulating GBP-1 may correlate with the IC levels that are present in the blood of the patients. This qualifies GBP-1 as a novel serological accessible marker to detect inflammatory activation mainly of micro- and macro-vascular EC *in vivo*.

During inflammation IC concentrations are increased. However, IC are often instable in the blood and are difficult to detect in tissues and are present in many different cells. Therefore, monitoring the inflammatory EC activation in inflammatory disease *via* the determination of circulating cytokine concentrations is not possible (Chen*, et al.* 1999; Park*, et al.* 2001; Mizia-Stec*, et al.* 2002).

An other soluble marker used to detect activation of EC is the intracellular adhesion molecule-1 (ICAM-1), which is the major ligand on EC for leukocytes. It has been shown that

the concentrations of soluble ICAM-1 (sICAM-1) are increased in a number of pathological states, including inflammation and sepsis (Sessler*, et al.* 1995; Ogawa*, et al.* 2000). sICAM-1 is a marker that indicates IC-activation of EC. In contrast to GBP-1, sICAM is upregulated not only by IC, but also by VEGF (Lu*, et al.* 1999; Kim*, et al.* 2001). In addition, sICAM is a mediator for the adhesion of different cells that in turn leads to an angiogenic activity of sICAM-1 (Gho*, et al.* 1999). Therefore, detection of sICAM does not indicate whether EC were activated by IC or VEGF and therefore also does not discriminate between the proliferative and non- proliferative phenotype.

Also Thy-1 has been presented as a soluble marker for EC activation (Saalbach*, et al.* 1999). However, not only EC, but also fibroblasts can be the source of soluble Thy-1 (Saalbach*, et al.* 1999). Therefore Thy-1 detected in the serum does not specifically indicate activation of EC.

Von Willebrand factor (vWF) is also used as a marker suggestive of damage/injury of the endothelium (Blann 1991; McGregor*, et al.* 1994). Increased concentrations of vWF have been detected for example in patients with sepsis (Wanecek*, et al.* 2000; Reinhart*, et al.* 2002). Secretion of stored vWF is increased by IL-1β. Whereas IFN-γ and TNF-α inhibit the release of vWF from EC (Tannenbaum*, et al.* 1990). In addition, vWF expression is up-regulated in EC by angiogenic factors like bFGF and VEGF (Zanetta*, et al.* 2000). Therefore vWF cannot discriminate between endothelium damage caused by IC or AGF.

Thrombospondin (TSP) is a further marker for inflammation. TSP secretion has been shown to be up-regulated in glomerulopathies (Okamoto*, et al.* 2002). TSP production by EC is decreased by treatment of the cells with IL-1β and TNF-α alone or in combination (Morandi*, et al.* 1994). By contrast, TSP has been shown to be up-regulated by growth factors like epidermal growth factor and transforming growth factor beta 1 (Morandi*, et al.* 1994). Therefore the increased circulating TSP cannot be used a marker for IC-activated EC, as in EC its production has been shown to be down-regulated by IC.

Finally, EC under shear stress secret endothelin-1 (Wang*, et al.* 2002). Shear-stress has been shown to activate EC and to inhibit expression of genes related to EC proliferation (Chen*, et al.* 2001; McCormick*, et al.* 2001). Micro-vascular EC increase endothelin-1 secretion under intermediate levels of shear stress, while macro-vascular EC decrease endothelin-1 secretion (McCormick*, et al.* 2001; Wang*, et al.* 2002). Therefore, changes in endothelin-1 secretion can be difficult to interpret because micro- and macro-vascular EC are reacting differently under the same conditions.

The use of GBP-1 as a marker of activated EC has many advantages. GBP-1 indicates selectively IC-activation of EC. GBP-1 can be used to detect temporal (on the tissue and/or on the serological level) and spatial (on the tissue level) appearance of IC-activated EC. GBP-1 indicates IC-activation also of micro-vascular EC that play an important role in wound healing or inflammatory diseases (Cotran*, et al.* 1988; Cotran*, et al.* 1990; Kapp 1993; Ensoli*, et al.;* 2000Yawalkar*, et al.* 2000). GBP-1 can also indicate IC-activation of macro-vascular EC, that has been shown to occur in the first phases of atherosclerosis (Cascieri 2002).

6 Possible role of secreted GBP-1

The role of secreted GBP-1 has still to be clarified. Of note, GBP-1 is a large GTPase. The GTPase domain could play a role in the function of extracellular GBP-1 by hydrolysing extracellular nucleotides (Cheng*, et al.* 1983; Cheng*, et al.* 1985; Boehm*, et al.* 1998; Schwemmle and Staeheli 1994; Prakash *et al.* 2000).

Extracellular nucleotides regulate a number of activities in many different cells. For example ATP triggers IL-1β and TNF-α release of macrophages and GTP regulates Ca^{2+} release and proliferation of B-lymphocytes (Di Virgilio*, et al.* 2001). Interestingly, in platelets it has been shown that ATP and GTP can often exert similar effects because they can bind to the same receptor (Greco*, et al.* 1992). In addition, it has been shown that GTP (but not GDP or GMP) enhances neurit outgrowth (Neary*, et al.* 1996). It has been suggested that an ecto-GTPase, may regulate inactivation of this process. Moreover, in trauma or ischemia increased concentrations of GTP and ATP were found to remain elevated for days (Neary*, et al.* 1996).

It is intriguing to speculate that GBP-1 may be such a postulated ecto-GTPase.

In addition, a growing family of nucleotide receptors has been described [reviewed in (Di Virgilio*, et al.* 2001)]. Receptors for purines are called P2 purinergic receptors. In mammalian twelve P2 receptors are known until now. All immune and inflammatory cells express P2 receptors (Di Virgilio*, et al.* 2001). In addition, purinergic receptors have been detected on EC (Neary*, et al.* 1996; von Albertini*, et al.* 1997). EC in addition express also ATP diphosphohydrolases that hydrolyse extracellular ATP adjusting the local ATP concentrations at low levels (Robson*, et al.* 1997). After activation, EC are no more antithrombotic (see Introduction, paragraph **1**) and increase their adhesiveness for blood cells. In addition EC loose the ATP diphosphohydrolases activity. As a consequence, extracellular ATP concentrations increase and induce adhesion of blood cells to EC. It has been shown that ATP induces E-selectin expression in EC (von Albertini*, et al.* 1997). This indicates that

nucleotide-mediated signalling may have an important role in the regulation of EC activation during inflammation.

GBP-1 has a high turnover GTPase activity and binds GTP with low affinity; in addition to GTP, it also binds GDP and GMP (Schwemmle*, et al.* 1994; Neun*, et al.* 1996; Praefcke*, et al.* 1999; van der Bliek 1999; Prakash*, et al.* 2000b). In this context extracellular GBP-1 may play a role in inflammation by regulating the extracellular GTP pool. In this framework, the results described here may define a platform to investigate novel signalling mechanisms in inflammatory diseases.

REFERENCES

Accola MA, Huang B, Al Masri A and McNiven MA (2002). The antiviral dynamin family member, MxA, tubulates lipids and localizes to the smooth endoplasmic reticulum. *J Biol Chem* **277** (24), 21829-35.

Ackermann L, Harvima IT, Pelkonen J, Ritamaki-Salo V, Naukkarinen A, Harvima RJ and Horsmanheimo M (1999). Mast cells in psoriatic skin are strongly positive for interferon-gamma. *Br J Dermatol* **140** (4), 624-33.

Aebi M, Fah J, Hurt N, Samuel CE, Thomis D, Bazzigher L, Pavlovic J, Haller O and Staeheli P (1989). cDNA structures and regulation of two interferon-induced human Mx proteins. *Mol Cell Biol* **9** (11), 5062-72

Alexander RW and Dzau VJ (2000). Vascular biology: the past 50 years. *Circulation* **102** (20 Suppl 4), IV112-6

Allan VJ, Thompson HM and McNiven MA (2002). Motoring around the Golgi. *Nat Cell Biol* **4** (10), E236-42

Anderson SL, Carton JM, Lou J, Xing L and Rubin BY (1999). Interferon-induced guanylate binding protein-1 (GBP-1) mediates an antiviral effect against vesicular stomatitis virus and encephalomyocarditis virus. *Virology* **256** (1), 8-14

Asundi VK, Stahl RC, Showalter L, Conner KJ and Carey DJ (1994). Molecular cloning and characterization of an isoprenylated 67 kDa protein. *Biochim Biophys Acta* **1217** (3), 257-65.

Augustin HG, Kozian DH and Johnson RC (1994). Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes. *Bioessays* **16** (12), 901-6.

Bachetti T and Morbidelli L (2000). Endothelial cells in culture: a model for studying vascular functions. *Pharmacol Res* **42** (1), 9-19

Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A and Marme D (1996). Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* **87** (8), 3336-43

Baumgartl HJ and Standl E (2001). The atherosclerotic process and its exacerbation by diabetes. *Exp Clin Endocrinol Diabetes* **109(Suppl** (2)), S487-S492.

Bechard D, Meignin V, Scherpereel A, Oudin S, Kervoaze G, Bertheau P, Janin A, Tonnel A and Lassalle P (2000). Characterization of the secreted form of endothelial-cell-specific molecule 1 by specific monoclonal antibodies. *J Vasc Res* **37** (5), 417-25

Beuscher HU, Gunther C and Rollinghoff M (1990). IL-1 beta is secreted by activated murine macrophages as biologically inactive precursor. *J Immunol* **144** (6), 2179-83

Bevilacqua MP, Stengelin S, Gimbrone MA, Jr. and Seed B (1989). Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* **243** (4895), 1160-5

Biedermann BC (2001). Vascular endothelium: checkpoint for inflammation and immunity. *News Physiol Sci* **16** 84-8

Black RA, Kronheim SR and Sleath PR (1989). Activation of interleukin-1 beta by a co-induced protease. *FEBS Lett* **247** (2), 386-90

Blann AD (1991). von Willebrand factor antigen as an acute phase reactant and marker of endothelial cell injury in connective tissue diseases: a comparison with CRP, rheumatoid factor, and erythrocyte sedimentation rate. *Z Rheumatol* **50** (5), 320-2

Boehm U, Guethlein L, Klamp T, Ozbek K, Schaub A, Futterer A, Pfeffer K and Howard JC (1998). Two families of GTPases dominate the complex cellular response to IFN- gamma. *J Immunol* **161** (12), 6715-23.

Breier G, Albrecht U, Sterrer S and Risau W (1992). Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* **114** (2), 521-32

Breuer-McHam JN, Marshall GD, Lewis DE and Duvic M (1998). Distinct serum cytokines in AIDS-related skin diseases. *Viral Immunol* 11 (4), 215-20

Brooks JJ (1986). Kaposi's sarcoma: a reversible hyperplasia. *Lancet* **2** (8519), 1309-11

Brown LF, Yeo KT, Berse B, Yeo TK, Senger DR, Dvorak HF and van de Water L (1992). Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med* **176** (5), 1375-9

Caldwell GA, Wang SH, Naider F and Becker JM (1994). Consequences of altered isoprenylation targets on a-factor export and bioactivity. *Proc Natl Acad Sci U S A* **91** (4), 1275-9

Cattoretti G, Becker MH, Key G, Duchrow M, Schluter C, Galle J and Gerdes J (1992). Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* **168** (4), 357-63

Carmeliet P and Jain RK (2000). Angiogenesis in cancer and other diseases. *Nature* **407** (6801), 249-57.

Cascieri MA (2002). The potential for novel anti-inflammatory therapies for coronary artery disease. *Nat Rev Drug Discov* **1** (2), 122-30

Cavender DE, Edelbaum D and Welkovich L (1991). Effects of inflammatory cytokines and phorbol esters on the adhesion of U937 cells, a human monocyte-like cell line, to endothelial cell monolayers and extracellular matrix proteins. *J Leukoc Biol* **49** (6), 566-78

Chang HC, Samaniego F, Nair BC, Buonaguro L and Ensoli B (1997). HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. *Aids* **11** (12), 1421-31.

Chardin P and McCormick F (1999). Brefeldin A: the advantage of being uncompetitive. *Cell* **97** (2), 153-5

Chen CC, Wang SS, Lee FY, Chang FY and Lee SD (1999). Proinflammatory cytokines in early assessment of the prognosis of acute pancreatitis. *Am J Gastroenterol* **94** (1), 213-8

Cheng YS, Becker-Manley MF, Chow TP and Horan DC (1985). Affinity purification of an interferon-induced human guanylate-binding protein and its characterization. *J Biol Chem* **260** (29), 15834-9.

Cheng YS, Colonno RJ and Yin FH (1983). Interferon induction of fibroblast proteins with guanylate binding activity. *J Biol Chem* **258** (12), 7746-50

Chodak GW, Hospelhorn V, Judge SM, Mayforth R, Koeppen H and Sasse J (1988). Increased levels of fibroblast growth factor-like activity in urine from patients with bladder or kidney cancer. *Cancer Res* **48** (8), 2083-8

Chodorowska G (1998). Plasma concentrations of IFN-gamma and TNF-alpha in psoriatic patients before and after local treatment with dithranol ointment. *J Eur Acad Dermatol Venereol* **10** (2), 147-51

Chopra V, Dinh TV and Hannigan EV (1998). Circulating serum levels of cytokines and angiogenic factors in patients with cervical cancer. *Cancer Invest* **16** (3), 152-9

Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, Ivanov IE and Philips MR (1999). Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* **98** (1), 69-80

Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM and Stern DM (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* **91** (10), 3527-61.

Cooper MA, Fehniger TA, Ponnappan A, Mehta V, Wewers MD and Caligiuri MA (2001). Interleukin-1beta costimulates interferon-gamma production by human natural killer cells. *Eur J Immunol* **31** (3), 792-801

Cornali E, Zietz C, Benelli R, Weninger W, Masiello L, Breier G, Tschachler E, Albini A and Stürzl M (1996). Vascular endothelial growth factor regulates angiogenesis and vascular permeability in Kaposi's sarcoma. *Am J Pathol* **149** (6), 1851-69.

Cotran RS and Pober JS (1988). Endothelial activation: its role in inflammatory and immune reactions. Plenum Press. 335-347
Cotran RS and Pober JS (1990). Cytokine-endothelial interactions in inflammation, immunity, and vascular injury. *J Am Soc Nephrol* **1** (3), 225-35.

Cozzolino F, Torcia M, Aldinucci D, Ziche M, Almerigogna F, Bani D and Stern DM (1990). Interleukin 1 is an autocrine regulator of human endothelial cell growth. *Proc Natl Acad Sci U S A* **87** (17), 6487-91

Dai Q, Choy E, Chiu V, Romano J, Slivka SR, Steitz SA, Michaelis S and Philips MR (1998). Mammalian prenylcysteine carboxyl methyltransferase is in the endoplasmic reticulum. *J Biol Chem* **273** (24), 15030-4

Danino D and Hinshaw JE (2001). Dynamin family of mechanoenzymes. *Curr Opin Cell Biol* **13** (4), 454-60

Datta YH and Ewenstein BM (2001). Regulated secretion in endothelial cells: biology and clinical implications. *Thromb Haemost* **86** (5), 1148-55

Decker T, Lew DJ, Cheng YS, Levy DE and Darnell JE, Jr. (1989). Interactions of alpha- and gamma-interferon in the transcriptional regulation of the gene encoding a guanylate-binding protein. *Embo J* **8** (7), 2009-14.

Desai SB and Libutti SK (1999). Tumor angiogenesis and endothelial cell modulatory factors. *J Immunother* **22** (3), 186-211

Detmar M, Brown LF, Schon MP, Elicker BM, Velasco P, Richard L, Fukumura D, Monsky W, Claffey KP and Jain RK (1998). Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J Invest Dermatol* **111** (1), 1-6

Diatloff-Zito C, Gordon AJ, Duchaud E and Merlin G (1995). Isolation of an ubiquitously expressed cDNA encoding human dynamin II, a member of the large GTP-binding protein family. *Gene* **163** (2), 301-6

Dinarello CA (1996). Biologic basis for interleukin-1 in disease. *Blood* **87** (6), 2095-147

Dinarello CA (2000). The role of the interleukin-1-receptor antagonist in blocking inflammation mediated by interleukin-1. *N Engl J Med* **343** (10), 732-4.

Dirix LY, Vermeulen PB, Pawinski A, Prove A, Benoy I, De Pooter C, Martin M and Van Oosterom AT (1997). Elevated levels of the angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in sera of cancer patients. *Br J Cancer* **76** (2), 238-43

Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, Torboli M, Bolognesi G and Baricordi OR (2001). Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* **97** (3), 587-600.

Donato R (2001). S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol* **33** (7), 637-68

Dower SK, Qwarnstrom EE, Page RC, Blanton RA, Kupper TS, Raines E, Ross R and Sims JE (1990). Biology of the interleukin-1 receptor. *J Invest Dermatol* **94** (6 Suppl), 68S-73S

Dzenko KA, Andjelkovic AV, Kuziel WA and Pachter JS (2001). The chemokine receptor CCR2 mediates the binding and internalization of monocyte chemoattractant protein-1 along brain microvessels. *J Neurosci* **21** (23), 9214-23.

Eccleston JF, Binns DD, Davis CT, Albanesi JP and Jameson DM (2002). Oligomerization and kinetic mechanism of the dynamin GTPase. *Eur Biophys J* **31** (4), 275-82

Eggermont AM (2002). Critical appraisal of IFN-alpha-based adjuvant therapy in stage II-III malignant melanoma. *Expert Rev Anticancer Ther* **2** (5), 563-9

Emilie D, Peuchmaur M, Maillot MC, Crevon MC, Brousse N, Delfraissy JF, Dormont J and Galanaud P (1990). Production of interleukins in human immunodeficiency virus-1-replicating lymph nodes. *J Clin Invest* **86** (1), 148-59

Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, Wingfield P and Gallo RC (1993). Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J Virol* **67** (1), 277-87.
Ensoli B, Markham P, Kao V, Barillari G, Fiorelli V, Gendelman R, Raffeld M, Zon G and Gallo RC (1994). Block of AIDS-Kaposi's sarcoma (KS) cell growth, angiogenesis, and lesion formation in nude mice by antisense oligonucleotide targeting basic fibroblast growth factor. A novel strategy for the therapy of KS. *J Clin Invest* **94** (5), 1736-46

Ensoli B, Sgadari C, Barillari G, Sirianni MC, Stürzl M and Monini P (2000). Biology of Kaposi's sarcoma. *Eur J Cancer* **37** (10), 1251-69.

Ensoli B and Stürzl M (1998). Kaposi's sarcoma: a result of the interplay among inflammatory cytokines, angiogenic factors and viral agents. *Cytokine Growth Factor Rev* **9** (1), 63-83

Esser S, Wolburg K, Wolburg H, Breier G, Kurzchalia T and Risau W (1998). Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J Cell Biol* **140** (4), 947-59

Fan TP, Jaggar R and Bicknell R (1995). Controlling the vasculature: angiogenesis, anti-angiogenesis and vascular targeting of gene therapy. *Trends Pharmacol Sci* **16** (2), 57-66

Farrar MA and Schreiber RD (1993). The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol 11 571-611

Feit J, Jedlickova H, Vlasin Z, Kempf W and Matyska L.
Atlas of dermatology. http://atlases.muni.cz/atl_en/sect_main.html

Ferrara N and Henzel WJ (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* **161** (2), 851-8.

Fiorelli V, Gendelman R, Sirianni MC, Chang HK, Colombini S, Markham PD, Monini P, Sonnabend J, Pintus A, Gallo RC and Ensoli B (1998). gamma-Interferon produced by CD8+ T cells infiltrating Kaposi's sarcoma induces spindle cells with angiogenic phenotype and synergy with human immunodeficiency virus-1 Tat protein: an immune response to human herpesvirus-8 infection? *Blood* **91** (3), 956-67.

Fisher C, Gilbertson-Beadling S, Powers EA, Petzold G, Poorman R and Mitchell MA (1994). Interstitial collagenase is required for angiogenesis in vitro. *Dev Biol* **162** (2), 499-510

Folkman J (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1** (1), 27-31

Folkman J and Hanahan D (1991). Switch to the angiogenic phenotype during tumorigenesis. *Princess Takamatsu Symp* **22** 339-47

Folkman J and Klagsbrun M (1987). Angiogenic factors. *Science* **235** (4787), 442-7.

Frater-Schroder M, Risau W, Hallmann R, Gautschi P and Bohlen P (1987). Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proc Natl Acad Sci U S A* **84** (15), 5277-81.

Friesel R, Komoriya A and Maciag T (1987). Inhibition of endothelial cell proliferation by gamma-interferon. *J Cell Biol* **104** (3), 689-96

Friesel R and Maciag T (1999). Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling. *Thromb Haemost* **82** (2), 748-54

Fu HW and Casey PJ (1999). Enzymology and biology of CaaX protein prenylation. *Recent Prog Horm Res* **54** 315-42; discussion 342-3

Fujisaki K, Mitsuyama K, Toyonaga A, Matsuo K and Tanikawa K (1998). Circulating vascular endothelial growth factor in patients with colorectal cancer. *Am J Gastroenterol* **93** (2), 249-52

Gentilini G, Kirschbaum NE, Augustine JA, Aster RH and Visentin GP (1999). Inhibition of human umbilical vein endothelial cell proliferation by the CXC chemokine, platelet factor 4 (PF4), is associated with impaired downregulation of p21(Cip1/WAF1). *Blood* **93** (1), 25-33.

Gho YS, Kleinman HK and Sosne G (1999). Angiogenic activity of human soluble intercellular adhesion molecule-1. *Cancer Res* **59** (20), 5128-32

Girard JP, Baekkevold ES, Yamanaka T, Haraldsen G, Brandtzaeg P and Amalric F (1999). Heterogeneity of endothelial cells: the specialized phenotype of human high endothelial venules characterized by suppression subtractive hybridization. *Am J Pathol* **155** (6), 2043-55

Gomi T, Shiohara T, Munakata T, Imanishi K and Nagashima M (1991). Interleukin 1 alpha, tumor necrosis factor alpha, and interferon gamma in psoriasis. *Arch Dermatol* **127** (6), 827-30

Goodbourn S, Didcock L and Randall RE (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *J Gen Virol* **81 Pt 10** 2341-64.

Gospodarowicz D (1984). Biological activity in vivo and in vitro of pituitary and brain fibroblast growth factor. *Symp Fundam Cancer Res* **37** 109-34

Gospodarowicz D (1991). Biological activities of fibroblast growth factors. *Ann N Y Acad Sci* **638** 1-8

Gottlieb AB, Luster AD, Posnett DN and Carter DM (1988). Detection of a gamma interferon-induced protein IP-10 in psoriatic plaques. *J Exp Med* **168** (3), 941-8.

Greco NJ, Tandon NN, Jackson BW and Jamieson GA (1992). Low structural specificity for nucleoside triphosphates as antagonists of ADP-induced platelet activation. *J Biol Chem* **267** (5), 2966-70.

Grimmond S, Lagercrantz J, Drinkwater C, Silins G, Townson S, Pollock P, Gotley D, Carson E, Rakar S, Nordenskjold M, Ward L, Hayward N and Weber G (1996). Cloning and characterization of a novel human gene related to vascular endothelial growth factor. *Genome Res* **6** (2), 124-31

Guenzi E, Töpolt K, Cornali E, Lubeseder-Martellato C, Jörg A, Matzen K, Zietz C, Kremmer E, Nappi F, Schwemmle M, Hohenadl C, Barillari G, Tschachler E, Monini P, Ensoli B and Stürzl M (2001). The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *Embo J* **20** (20), 5568-77.

Haller O and Kochs G (2002). Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. *Traffic* **3** (10), 710-7

Haraldsen G, Kvale D, Lien B, Farstad IN and Brandtzaeg P (1996). Cytokine-regulated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human microvascular endothelial cells. *J Immunol* **156** (7), 2558-65.

Hari Y, Urwyler A, Hurni M, Yawalkar N, Dahinden C, Wendland T, Braathen LR, Matter L and Pichler WJ (1999). Distinct serum cytokine levels in drug- and measles-induced exanthema. *Int Arch Allergy Immunol* **120** (3), 225-9.

Harlow E, Lane, D. (1999). Using antibodies: a laboratory manual. Cold Spring Harbor Laboratory press.

Harrison VJ, Barnes K, Turner AJ, Wood E, Corder R and Vane JR (1995). Identification of endothelin 1 and big endothelin 1 in secretory vesicles isolated from bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* **92** (14), 6344-8

Harter C and Reinhard C (2000). The secretory pathway from history to the state of the art. *Subcell Biochem* **34** 1-38

Hayward CP, Cramer EM, Song Z, Zheng S, Fung R, Masse JM, Stead RH and Podor TJ (1998). Studies of multimerin in human endothelial cells. *Blood* **91** (4), 1304-17

Hayward CP, Song Z, Zheng S, Fung R, Pai M, Masse JM and Cramer EM (1999). Multimerin processing by cells with and without pathways for regulated protein secretion. *Blood* **94** (4), 1337-47

Helms JB, Helms-Brons D, Brugger B, Gkantiragas I, Eberle H, Nickel W, Nurnberg B, Gerdes HH and Wieland FT (1998). A putative heterotrimeric G protein inhibits the fusion of COPI-coated vesicles. Segregation of heterotrimeric G proteins from COPI-coated vesicles. *J Biol Chem* **273** (24), 15203-8

Hiraoka N, Allen E, Apel IJ, Gyetko MR and Weiss SJ (1998). Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell* **95** (3), 365-77
Hisadome K, Koyama T, Kimura C, Droogmans G, Ito Y and Oike M (2002). Volume-regulated Anion Channels Serve as an Auto/Paracrine Nucleotide Release Pathway in Aortic Endothelial Cells. *J Gen Physiol* **119** (6), 511-20.

Hober D, Haque A, Wattre P, Beaucaire G, Mouton Y and Capron A (1989). Production of tumour necrosis factor-alpha (TNF-alpha) and interleukin-1 (IL-1) in patients with AIDS. Enhanced level of TNF-alpha is related to a higher cytotoxic activity. *Clin Exp Immunol* **78** (3), 329-33

Hochuli E, Dobeli H and Schacher A (1987). New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J Chromatogr* **411** 177-84

Hofemeister H, Weber K and Stick R (2000). Association of prenylated proteins with the plasma membrane and the inner nuclear membrane is mediated by the same membrane-targeting motifs. *Mol Biol Cell* **11** (9), 3233-46

Holzinger C, Weissinger E, Zuckermann A, Imhof M, Kink F, Schollhammer A, Kopp C and Wolner E (1993). Effects of interleukin-1, -2, -4, -6, interferon-gamma and granulocyte/macrophage colony stimulating factor on human vascular endothelial cells. *Immunol Lett* **35** (2), 109-17.

Huang XL, Fan Z, Kalinyak C, Mellors JW and Rinaldo CR, Jr. (2000). CD8(+) T-cell gamma interferon production specific for human immunodeficiency virus type 1 (HIV-1) in HIV-1-infected subjects. *Clin Diagn Lab Immunol* **7** (2), 279-87

Jackson A, Tarantini F, Gamble S, Friedman S and Maciag T (1995). The release of fibroblast growth factor-1 from NIH 3T3 cells in response to temperature involves the function of cysteine residues. *J Biol Chem* **270** (1), 33-6.

Jaramillo ML, Abraham N and Bell JC (1995). The interferon system: a review with emphasis on the role of PKR in growth control. *Cancer Invest* **13** (3), 327-38

Joiner KA and Roos DS (2002). Secretory traffic in the eukaryotic parasite Toxoplasma gondii: less is more. *J Cell Biol* **157** (4), 557-63

Jung U and Ley K (1997). Regulation of E-selectin, P-selectin, and intercellular adhesion molecule 1 expression in mouse cremaster muscle vasculature. *Microcirculation* **4** (2), 311-9.

Jung U, Norman KE, Scharffetter-Kochanek K, Beaudet AL and Ley K (1998). Transit time of leukocytes rolling through venules controls cytokine- induced inflammatory cell recruitment in vivo. *J Clin Invest* **102** (8), 1526-33.

Kaminishi K, Soma Y, Kawa Y and Mizoguchi M (2002). Flow cytometric analysis of IL-4, IL-13 and IFN-gamma expression in peripheral blood mononuclear cells and detection of circulating IL-13 in patients with atopic dermatitis provide evidence for the involvement of type 2 cytokines in the disease. *J Dermatol Sci* **29** (1), 19-25

Kaplanski G, Fabrigoule M, Boulay V, Dinarello CA, Bongrand P, Kaplanski S and Farnarier C (1997). Thrombin induces endothelial type II activation in vitro: IL-1 and TNF-alpha-independent IL-8 secretion and E-selectin expression. *J Immunol* **158** (11), 5435-41

Kapp A (1993). The role of cytokines in the psoriatic inflammation. *J Dermatol Sci* **5** (3), 133-42.

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J and Connolly DT (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* **246** (4935), 1309-12.

Kim I, Moon SO, Kim SH, Kim HJ, Koh YS and Koh GY (2001). Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E- selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem* **276** (10), 7614-20.

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS and Ferrara N (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* **362** (6423), 841-4

Kirchhausen T (2000). Three ways to make a vesicle. *Nat Rev Mol Cell Biol* **1** (3), 187-98

Klein S, Giancotti FG, Presta M, Albelda SM, Buck CA and Rifkin DB (1993). Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol Biol Cell* **4** (10), 973-82

Krishnaswamy G, Kelley J, Yerra L, Smith JK and Chi DS (1999). Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease. *J Interferon Cytokine Res* **19** (2), 91-104.

Kuchler K, Sterne RE and Thorner J (1989). Saccharomyces cerevisiae STE6 gene product: a novel pathway for protein export in eukaryotic cells. *Embo J* **8** (13), 3973-84

Kumar S, Li Q, Dua A, Ying YK, Bagchi MK and Bagchi IC (2001). Messenger ribonucleic acid encoding interferon-inducible guanylate binding protein 1 is induced in human endometrium within the putative window of implantation. *J Clin Endocrinol Metab* **86** (6), 2420-7.

Kuzu I, Bicknell R, Harris AL, Jones M, Gatter KC and Mason DY (1992). Heterogeneity of vascular endothelial cells with relevance to diagnosis of vascular tumours. *J Clin Pathol* **45** (2), 143-8

Lambert A, Sadir R, Brisson C and Morel G (2000). In vivo subcellular target compartments of interferon-gamma and interferon-gamma receptor (alpha- and beta-chains) in mouse liver. *Cytokine* **12** (6), 715-9.

Landriscina M, Cassano A, Ratto C, Longo R, Ippoliti M, Palazzotti B, Crucitti F and Barone C (1998). Quantitative analysis of basic fibroblast growth factor and vascular endothelial growth factor in human colorectal cancer. *Br J Cancer* **78** (6), 765-70

Larrick JW and Kunkel SL (1988). The role of tumor necrosis factor and interleukin 1 in the immunoinflammatory response. *Pharm Res* **5** (3), 129-39

Last-Barney K, Homon CA, Faanes RB and Merluzzi VJ (1988). Synergistic and overlapping activities of tumor necrosis factor-alpha and IL-1. *J Immunol* **141** (2), 527-30

Lee YW, Kuhn H, Hennig B, Neish AS and Toborek M (2001). IL-4-induced oxidative stress upregulates VCAM-1 gene expression in human endothelial cells. *J Mol Cell Cardiol* **33** (1), 83-94.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV and Ferrara N (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246** (4935), 1306-9

Ley K (1996). Molecular mechanisms of leukocyte recruitment in the inflammatory process. *Cardiovasc Res* **32** (4), 733-42.

Livni E, Lapidoth M and Halevy S (1999). In vitro release of interferon-gamma and macrophage migration inhibition factor in drug-induced urticaria and angioedema. *Acta Derm Venereol* **79** (1), 18-22

Luan Z, Zhang Y, Liu A, Man Y, Cheng L and Hu G (2002). A novel GTP-binding protein hGBP3 interacts with NIK/HGK. *FEBS Lett* **530** (1-3), 233-8

Lu M, Perez VL, Ma N, Miyamoto K, Peng HB, Liao JK and Adamis AP (1999). VEGF increases retinal vascular ICAM-1 expression in vivo. *Invest Ophthalmol Vis Sci* **40** (8), 1808-12

Lubeseder-Martellato C, Guenzi E, Jörg A, Töpolt K, Naschberger E, Kremmer E, Zietz C, Tschachler E, Hutzler P, Schwemmle M, Matzen K, Grimm T, Ensoli B and Stürzl M (2002). Guanylate-Binding Protein-1 Expression Is Selectively Induced by Inflammatory Cytokines and Is an Activation Marker of Endothelial Cells during Inflammatory Diseases. *Am J Pathol* **161** (5), 1749-59

Luscinskas FW, Kansas GS, Ding H, Pizcueta P, Schleiffenbaum BE, Tedder TF and Gimbrone MA, Jr. (1994). Monocyte rolling, arrest and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, beta 1-integrins, and beta 2-integrins. *J Cell Biol* **125** (6), 1417-27.

Madge LA and Pober JS (2001). TNF signaling in vascular endothelial cells. *Exp Mol Pathol* **70** (3), 317-25.

Maglione D, Guerriero V, Viglietto G, Delli-Bovi P and Persico MG (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci U S A* **88** (20), 9267-71

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN and Yancopoulos GD (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277** (5322), 55-60.

Mallat Z, Corbaz A, Scoazec A, Besnard S, Leseche G, Chvatchko Y and Tedgui A (2001). Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation* **104** (14), 1598-603.

March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K and et al. (1985). Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* **315** (6021), 641-7

Maruo N, Morita I, Shirao M and Murota S (1992). IL-6 increases endothelial permeability in vitro. *Endocrinology* **131** (2), 710-4.

Maruyama I, Yonezawa S, Sato E and Igata A (1989). Endothelium and thrombomodulin: expression and distribution of thrombomodulin on altered endothelium and neoplastic syncytiotrophoblast. *Nippon Ketsueki Gakkai Zasshi* **52** (1), 150-5

Maxfield FR, Willingham MC, Davies PJ and Pastan I (1979). Amines inhibit the clustering of alpha2-macroglobulin and EGF on the fibroblast cell surface. *Nature* **277** (5698), 661-3.

Mason DY, Micklem K and Jones M (2000). Double immunofluorescence labelling of routinely processed paraffin sections. *J Pathol* **191** (4), 452-61.

Mason JC, Yarwood H, Sugars K and Haskard DO (1997). Human umbilical vein and dermal microvascular endothelial cells show heterogeneity in response to PKC activation. *Am J Physiol* **273** (4 Pt 1), C1233-40

May LT, Torcia G, Cozzolino F, Ray A, Tatter SB, Santhanam U, Sehgal PB and Stern D (1989). Interleukin-6 gene expression in human endothelial cells: RNA start sites, multiple IL-6 proteins and inhibition of proliferation. *Biochem Biophys Res Commun* **159** (3), 991-8

McCormick SM, Eskin SG, McIntire LV, Teng CL, Lu CM, Russell CG and Chittur KK (2001). DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. *Proc Natl Acad Sci U S A* **98** (16), 8955-60.

McGrath JP and Varshavsky A (1989). The yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature* **340** (6232), 400-4

McGregor B, Colon S, Mutin M, Chignier E, Zech P and McGregor J (1994). Thrombospondin in human glomerulopathies. A marker of inflammation and early fibrosis. *Am J Pathol* **144** (6), 1281-7

McMahon GA, Garfinkel S, Prudovsky I, Hu X and Maciag T (1997). Intracellular precursor interleukin (IL)-1alpha, but not mature IL-1alpha, is able to regulate human endothelial cell migration in vitro. *J Biol Chem* **272** (45), 28202-5

McNutt NS, Fletcher V and Conant MA (1983). Early lesions of Kaposi's sarcoma in homosexual men. An ultrastructural comparison with other vascular proliferations in skin. *Am J Pathol* **111** (1), 62-77

Melder RJ, Koenig GC, Witwer BP, Safabakhsh N, Munn LL and Jain RK (1996). During angiogenesis, vascular endothelial growth factor and basic fibroblast growth factor regulate natural killer cell adhesion to tumor endothelium. *Nat Med* **2** (9), 992-7

Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M, Lanz C, Buttner M, Rziha HJ and Dehio C (1999). A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *Embo J* **18** (2), 363-74

Michaelis S (1993). STE6, the yeast a-factor transporter. *Semin Cell Biol* **4** (1), 17-27

Misumi Y, Miki K, Takatsuki A, Tamura G and Ikehara Y (1986). Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J Biol Chem* **261** (24), 11398-403

Mizia-Stec K, Mandecki T, Zahorska-Markiewicz B, Janowska J, Szulc A, Jastrzebska-Maj E, Szymanski L and Majewski T (2002). Selected cytokines and soluble forms of cytokine receptors in coronary artery disease. **13** (2), 115-122

Montesano R, Vassalli JD, Baird A, Guillemin R and Orci L (1986). Basic fibroblast growth factor induces angiogenesis in vitro. *Proc Natl Acad Sci U S A* **83** (19), 7297-301

Montrucchio G, Lupia E, Battaglia E, Passerini G, Bussolino F, Emanuelli G and Camussi G (1994). Tumor necrosis factor alpha-induced angiogenesis depends on in situ platelet-activating factor biosynthesis. *J Exp Med* **180** (1), 377-82

Moore KW, de Waal Malefyt R, Coffman RL and O'Garra A (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19** 683-765

Morandi V, Cherradi SE, Lambert S, Fauvel-Lafeve F, Legrand YJ and Legrand C (1994). Proinflammatory cytokines (interleukin-1 beta and tumor necrosis factor-alpha) down regulate synthesis and secretion of thrombospondin by human endothelial cells. *J Cell Physiol* **160** (2), 367-77

Moscatelli D, Presta M and Rifkin DB (1986). Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc Natl Acad Sci U S A* **83** (7), 2091-5

Moses MA (1997). The regulation of neovascularization of matrix metalloproteinases and their inhibitors. *Stem Cells* **15** (3), 180-9

Mosley B, Urdal DL, Prickett KS, Larsen A, Cosman D, Conlon PJ, Gillis S and Dower SK (1987). The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. *J Biol Chem* **262** (7), 2941-4

Murdoch C, Monk PN and Finn A (1999). Cxc chemokine receptor expression on human endothelial cells. *Cytokine* **11** (9), 704-12.

Nakamoto T, Chang CS, Li AK and Chodak GW (1992). Basic fibroblast growth factor in human prostate cancer cells. *Cancer Res* **52** (3), 571-7

Nantais DE, Schwemmle M, Stickney JT, Vestal DJ and Buss JE (1996). Prenylation of an interferon-gamma-induced GTP-binding protein: the human guanylate binding protein, huGBP1. *J Leukoc Biol* **60** (3), 423-31

Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP and Burnstock G (1996). Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci* **19** (1), 13-8.

Nedwin GE, Svedersky LP, Bringman TS, Palladino MA, Jr. and Goeddel DV (1985). Effect of interleukin 2, interferon-gamma, and mitogens on the production of tumor necrosis factors alpha and beta. *J Immunol* **135** (4), 2492-7

Neufeld G, Cohen T, Gengrinovitch S and Poltorak Z (1999). Vascular endothelial growth factor (VEGF) and its receptors. *Faseb J* **13** (1), 9-22

Neufeld G, Cohen T, Gitay-Goren H, Poltorak Z, Tessler S, Sharon R, Gengrinovitch S and Levi BZ (1996). Similarities and differences between the vascular endothelial growth factor (VEGF) splice variants. *Cancer Metastasis Rev* **15** (2), 153-8

Neun R, Richter MF, Staeheli P and Schwemmle M (1996). GTPase properties of the interferon-induced human guanylate-binding protein 2. *FEBS Lett* **390** (1), 69-72.

Nguyen TT, Hu Y, Widney DP, Mar RA and Smith JB (2002). Murine GBP-5, a new member of the murine guanylate-binding protein family, is coordinately regulated with other GBPs in vivo and in vitro. *J Interferon Cytokine Res* **22** (8), 899-909

Nicosia RF, Nicosia SV and Smith M (1994). Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. *Am J Pathol* **145** (5), 1023-9

Nicosia RF, Tchao R and Leighton J (1983). Angiogenesis-dependent tumor spread in reinforced fibrin clot culture. *Cancer Res* **43** (5), 2159-66

Niles WD and Malik AB (1999). Endocytosis and exocytosis events regulate vesicle traffic in endothelial cells. *J Membr Biol* **167** (1), 85-101

Nilius B and Droogmans G (2001). Ion channels and their functional role in vascular endothelium. *Physiol Rev* **81** (4), 1415-59

Nophar Y, Kemper O, Brakebusch C, Englemann H, Zwang R, Aderka D, Holtmann H and Wallach D (1990). Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *Embo J* **9** (10), 3269-78

Norioka K, Mitaka T, Mochizuki Y, Hara M, Kawagoe M and Nakamura H (1994). Interaction of interleukin-1 and interferon-gamma on fibroblast growth factor-induced angiogenesis. *Jpn J Cancer Res* **85** (5), 522-9

Ogawa H, Sakamoto T, Nishiyama K, Soejima H, Kaikita K, Takazoe K, Miyamoto S, Kugiyama K, Yoshimura M and Yasue H (2000). Elevated levels of soluble intercellular adhesion molecule-1 in the coronary circulation of patients with coronary organic stenosis and spasm. *Jpn Circ J* **64** (3), 170-6

Okamoto M, Ono M, Uchiumi T, Ueno H, Kohno K, Sugimachi K and Kuwano M (2002). Up-regulation of thrombospondin-1 gene by epidermal growth factor and transforming growth factor beta in human cancer cells--transcriptional activation and messenger RNA stabilization. *Biochim Biophys Acta* **1574** (1), 24-34

Olofsson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, Orpana A, Pettersson RF, Alitalo K and Eriksson U (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci U S A* **93** (6), 2576-81

Pandita R, Pocsik E and Aggarwal BB (1992). Interferon-gamma induces cell surface expression for both types of tumor necrosis factor receptors. *FEBS Lett* **312** (1), 87-90

Park CC, Morel JC, Amin MA, Connors MA, Harlow LA and Koch AE (2001). Evidence of IL-18 as a novel angiogenic mediator. *J Immunol* **167** (3), 1644-53.

Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB and Goeddel DV (1984). Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* **312** (5996), 724-9

Pepper MS (2001). Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol* **21** (7), 1104-17

Pepper MS, Ferrara N, Orci L and Montesano R (1992). Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochem Biophys Res Commun* **189** (2), 824-31

Pestka S, Kotenko SV, Muthukumaran G, Izotova LS, Cook JR and Garotta G (1997). The interferon gamma (IFN-gamma) receptor: a paradigm for the multichain cytokine receptor. *Cytokine Growth Factor Rev* **8** (3), 189-206.

Phillips GD, Stone AM, Jones BD, Schultz JC, Whitehead RA and Knighton DR (1994). Vascular endothelial growth factor (rhVEGF165) stimulates direct angiogenesis in the rabbit cornea. *In Vivo* **8** (6), 961-5

Phillips HS, Hains J, Leung DW and Ferrara N (1990). Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology* **127** (2), 965-7

Pitts KR, Yoon Y, Krueger EW and McNiven MA (1999). The dynamin-like protein DLP1 is essential for normal distribution and morphology of the endoplasmic reticulum and mitochondria in mammalian cells. *Mol Biol Cell* **10** (12), 4403-17

Plate KH, Breier G, Weich HA and Risau W (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* **359** (6398), 845-8

Pober JS (1988). Warner-Lambert/Parke-Davis award lecture. Cytokine-mediated activation of vascular endothelium. Physiology and pathology. *Am J Pathol* **133** (3), 426-33.

Pober JS, Gimbrone MA, Jr., Lapierre LA, Mendrick DL, Fiers W, Rothlein R and Springer TA (1986). Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J Immunol* **137** (6), 1893-6.

Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, Keshet E and Neufeld G (1997). VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. *J Biol Chem* **272** (11), 7151-8

Praefcke GJ, Geyer M, Schwemmle M, Robert Kalbitzer H and Herrmann C (1999). Nucleotide-binding characteristics of human guanylate-binding protein 1 (hGBP1) and identification of the third GTP-binding motif. *J Mol Biol* **292** (2), 321-32

Prakash B, Praefcke GJ, Renault L, Wittinghofer A and Herrmann C (2000a). Structure of human guanylate-binding protein 1 representing a unique class of GTP-binding proteins. *Nature* **403** (6769), 567-71

Prakash B, Renault L, Praefcke GJ, Herrmann C and Wittinghofer A (2000b). Triphosphate structure of guanylate-binding protein 1 and implications for nucleotide binding and GTPase mechanism. *Embo J* **19** (17), 4555-64.

Prudovsky I, Bagala C, Tarantini F, Mandinova A, Soldi R, Bellum S and Maciag T (2002). The intracellular translocation of the components of the fibroblast growth factor 1 release complex precedes their assembly prior to export. *J Cell Biol* **158** (2), 201-8

Reinhart K, Bayer O, Brunkhorst F and Meisner M (2002). Markers of endothelial damage in organ dysfunction and sepsis. *Crit Care Med* **30** (5 Suppl), S302-12

Robson SC, Kaczmarek E, Siegel JB, Candinas D, Koziak K, Millan M, Hancock WW and Bach FH (1997). Loss of ATP diphosphohydrolase activity with endothelial cell activation. *J Exp Med* **185** (1), 153-63.

Roesen P, Ferber P and Tschoepe D (2001). Macrovascular disease in diabetes: current status. *Exp Clin Endocrinol Diabetes* **109(Suppl** (2)), S474-S486.

Romero LI, Zhang DN, Herron GS and Karasek MA (1997). Interleukin-1 induces major phenotypic changes in human skin microvascular endothelial cells. *J Cell Physiol* **173** (1), 84-92.

Roth WK, Werner S, Risau W, Remberger K and Hofschneider PH (1988). Cultured, AIDS-related Kaposi's sarcoma cells express endothelial cell markers and are weakly malignant in vitro. *Int J Cancer* **42** (5), 767-73

Rubartelli A, Cozzolino F, Talio M and Sitia R (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *Embo J* **9** (5), 1503-10.

Rubinstein M, Novick D and Fischer DG (1987). The human interferon-gamma receptor system. *Immunol Rev* **97** 29-50.

Ruszczak Z, Detmar M, Imcke E and Orfanos CE (1990). Effects of rIFN alpha, beta, and gamma on the morphology, proliferation, and cell surface antigen expression of human dermal microvascular endothelial cells in vitro. *J Invest Dermatol* **95** (6), 693-9

Ruoslahti E (2000). Targeting tumor vasculature with homing peptides from phage display. *Semin Cancer Biol* **10** (6), 435-42.

Saalbach A, Wetzig T, Haustein UF and Anderegg U (1999). Detection of human soluble Thy-1 in serum by ELISA. Fibroblasts and activated endothelial cells are a possible source of soluble Thy-1 in serum. *Cell Tissue Res* **298** (2), 307-15

Sadir R, Lortat-Jacob H and Morel G (2000). Internalization and nuclear translocation of IFN-gamma and IFN-gammaR: an ultrastructural approach. *Cytokine* **12** (6), 711-4.

Salahuddin SZ, Nakamura S, Biberfeld P, Kaplan MH, Markham PD, Larsson L and Gallo RC (1988). Angiogenic properties of Kaposi's sarcoma-derived cells after long-term culture in vitro. *Science* **242** (4877), 430-3

Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ and Murphy WJ (2000). Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* **96** (1), 34-40.

Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, Kleinman HK, Murphy WJ and Oppenheim JJ (1999). Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: In vivo neovascularization induced by stromal-derived factor-1alpha. *Am J Pathol* **154** (4), 1125-35.

Salven P, Lymboussaki A, Heikkila P, Jaaskela-Saari H, Enholm B, Aase K, von Euler G, Eriksson U, Alitalo K and Joensuu H (1998). Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. *Am J Pathol* **153** (1), 103-8

Samaniego F, Markham PD, Gallo RC and Ensoli B (1995). Inflammatory cytokines induce AIDS-Kaposi's sarcoma-derived spindle cells to produce and release basic fibroblast growth factor and enhance Kaposi's sarcoma-like lesion formation in nude mice. *J Immunol* **154** (7), 3582-92

Samaniego F, Markham PD, Gendelman R, Watanabe Y, Kao V, Kowalski K, Sonnabend JA, Pintus A, Gallo RC and Ensoli B (1998). Vascular endothelial growth factor and basic fibroblast growth factor present in Kaposi's sarcoma (KS) are induced by inflammatory cytokines and synergize to promote vascular permeability and KS lesion development. *Am J Pathol* **152** (6), 1433-43

Sanders VJ, Pittman CA, White MG, Wang G, Wiley CA and Achim CL (1998). Chemokines and receptors in HIV encephalitis. *Aids* **12** (9), 1021-6.

Sata M, Moss J and Vaughan M (1999). Structural basis for the inhibitory effect of brefeldin A on guanine nucleotide-exchange proteins for ADP-ribosylation factors. *Proc Natl Acad Sci U S A* **96** (6), 2752-7

Sato Y and Rifkin DB (1988). Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. *J Cell Biol* **107** (3), 1199-205

Saunders NA, Smith RJ and Jetten AM (1999). Regulation of guanylate-binding protein expression in interferon-gamma-treated human epidermal keratinocytes and squamous cell carcinoma cells. *J Invest Dermatol* **112** (6), 977-83

Schekman R (1998). Membrane fusion. Ready...aim...fire! *Nature* **396** (6711), 514-5

Schekman R (2002). Lasker Basic Medical Research Award. SEC mutants and the secretory apparatus. *Nat Med* **8** (10), 1055-8

Schekman R and Orci L (1996). Coat proteins and vesicle budding. *Science* **271** (5255), 1526-33

Schmidt WK, Tam A, Fujimura-Kamada K and Michaelis S (1998). Endoplasmic reticulum membrane localization of Rce1p and Ste24p, yeast proteases involved in carboxyl-terminal CAAX protein processing and amino-terminal a-factor cleavage. *Proc Natl Acad Sci U S A* **95** (19), 11175-80

Schreiber RD, Farrar MA, Hershey GK and Fernandez-Luna J (1992). The structure and function of interferon-gamma receptors. *Int J Immunopharmacol* **14** (3), 413-9.

Schweigerer L, Malerstein B and Gospodarowicz D (1987a). Tumor necrosis factor inhibits the proliferation of cultured capillary endothelial cells. *Biochem Biophys Res Commun* **143** (3), 997-1004

Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC and Gospodarowicz D (1987b). Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. *Nature* **325** (6101), 257-9

Schwemmle M and Staeheli P (1994). The interferon-induced 67-kDa guanylate-binding protein (hGBP1) is a GTPase that converts GTP to GMP. *J Biol Chem* **269** (15), 11299-305

Sessler CN, Windsor AC, Schwartz M, Watson L, Fisher BJ, Sugerman HJ and Fowler AA, 3rd (1995). Circulating ICAM-1 is increased in septic shock. *Am J Respir Crit Care Med* **151** (5), 1420-7

Shimizu Y, Newman W, Tanaka Y and Shaw S (1992). Lymphocyte interactions with endothelial cells. *Immunol Today* **13** (3), 106-12.

Shuai K, Liao J and Song MM (1996). Enhancement of antiproliferative activity of gamma interferon by the specific inhibition of tyrosine dephosphorylation of Stat1. *Mol Cell Biol* **16** (9), 4932-41.

Siegel G and Malmsten M (1997). The role of the endothelium in inflammation and tumor metastasis. *Int J Microcirc Clin Exp* **17** (5), 257-72

Silletti S, Kessler T, Goldberg J, Boger DL and Cheresh DA (2001). Disruption of matrix metalloproteinase 2 binding to integrin alpha vbeta 3 by an organic molecule inhibits angiogenesis and tumor growth in vivo. *Proc Natl Acad Sci U S A* **98** (1), 119-24

Simons K and Ikonen E (1997). Functional rafts in cell membranes. *Nature* **387** (6633), 569-72

Sirianni MC, Vincenzi L, Fiorelli V, Topino S, Scala E, Uccini S, Angeloni A, Faggioni A, Cerimele D, Cottoni F, Aiuti F and Ensoli B (1998). gamma-Interferon production in peripheral blood mononuclear cells and tumor infiltrating lymphocytes from Kaposi's sarcoma patients: correlation with the presence of human herpesvirus-8 in peripheral blood mononuclear cells and lesional macrophages. *Blood* **91** (3), 968-76

Soderberg A, Sahaf B and Rosen A (2000). Thioredoxin reductase, a redox-active selenoprotein, is secreted by normal and neoplastic cells: presence in human plasma. *Cancer Res* **60** (8), 2281-9.

Stewart TE, Valenza F, Ribeiro SP, Wener AD, Volgyesi G, Mullen JB and Slutsky AS (1995). Increased nitric oxide in exhaled gas as an early marker of lung inflammation in a model of sepsis. *Am J Respir Crit Care Med* **151** (3 Pt 1), 713-8

St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B and Kinzler KW (2000). Genes expressed in human tumor endothelium. *Science* **289** (5482), 1197-202

Stürzl M, Brandstetter H, Zietz C, Eisenburg B, Raivich G, Gearing DP, Brockmeyer NH and Hofschneider PH (1995). Identification of interleukin-1 and platelet-derived growth factor-B as major mitogens for the spindle cells of Kaposi's sarcoma: a combined in vitro and in vivo analysis. *Oncogene* **10** (10), 2007-16.

Stürzl M, Zietz C, Monini P and Ensoli B (2001). Human herpesvirus-8 and Kaposi's sarcoma: relationship with the multistep concept of tumorigenesis. *Adv Cancer Res* **81** 125-59

Swerlick RA and Lawley TJ (1993). Role of microvascular endothelial cells in inflammation. *J Invest Dermatol* **100** (1), 111S-115S.

Swerlick RA, Lee KH, Li LJ, Sepp NT, Caughman SW and Lawley TJ (1992). Regulation of vascular cell adhesion molecule 1 on human dermal microvascular endothelial cells. *J Immunol* **149** (2), 698-705.

Swinscoe JC and Carlson EC (1992). Capillary endothelial cells secrete a heparin-binding mitogen for pericytes. *J Cell Sci* **103 (Pt 2)** 453-61

Symons JA, Eastgate JA and Duff GW (1991). Purification and characterization of a novel soluble receptor for interleukin 1. *J Exp Med* **174** (5), 1251-4

Takashima S and Klagsbrun M (1996). Inhibition of endothelial cell growth by macrophage-like U-937 cell-derived oncostatin M, leukemia inhibitory factor, and transforming growth factor beta1. *J Biol Chem* **271** (40), 24901-6.

Tan P, Luscinskas FW and Homer-Vanniasinkam S (1999). Cellular and molecular mechanisms of inflammation and thrombosis. *Eur J Vasc Endovasc Surg* **17** (5), 373-89

Tang BL, Zhang T, Low DY, Wong ET, Horstmann H and Hong W (2000). Mammalian homologues of yeast sec31p. An ubiquitously expressed form is localized to endoplasmic reticulum (ER) exit sites and is essential for ER-Golgi transport. *J Biol Chem* **275** (18), 13597-604

Tang BL, Ong YS, Huang B, Wei S, Wong ET, Qi R, Horstmann H and Hong W (2001). A membrane protein enriched in endoplasmic reticulum exit sites interacts with COPII. *J Biol Chem* **276** (43), 40008-17

Tanimoto H, Yoshida K, Yokozaki H, Yasui W, Nakayama H, Ito H, Ohama K and Tahara E (1991). Expression of basic fibroblast growth factor in human gastric carcinomas. *Virchows Arch B Cell Pathol Incl Mol Pathol* **61** (4), 263-7

Tannenbaum SH and Gralnick HR (1990). Gamma-interferon modulates von Willebrand factor release by cultured human endothelial cells. *Blood* **75** (11), 2177-84

Taraboletti G, Sonzogni L, Vergani V, Hosseini G, Ceruti R, Ghilardi C, Bastone A, Toschi E, Borsotti P, Scanziani E, Giavazzi R, Pepper MS, Stetler-Stevenson WG and Bani MR (2000). Posttranscriptional stimulation of endothelial cell matrix metalloproteinases 2 and 1 by endothelioma cells. *Exp Cell Res* **258** (2), 384-94.

Tarantini F, Micucci I, Bellum S, Landriscina M, Garfinkel S, Prudovsky I and Maciag T (2001). The precursor but not the mature form of IL1alpha blocks the release of FGF1 in response to heat shock. *J Biol Chem* **276** (7), 5147-51

Terry CM, Clikeman JA, Hoidal JR and Callahan KS (1999). TNF-alpha and IL-1alpha induce heme oxygenase-1 via protein kinase C, Ca2+, and phospholipase A2 in endothelial cells. *Am J Physiol* **276** (5 Pt 2), H1493-501.

Tartakoff AM (1983). Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* **32** (4), 1026-8.

Thomas KA, Rios-Candelore M, Gimenez-Gallego G, DiSalvo J, Bennett C, Rodkey J and Fitzpatrick S (1985). Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1. *Proc Natl Acad Sci U S A* **82** (19), 6409-13

Thommen R, Humar R, Misevic G, Pepper MS, Hahn AW, John M and Battegay EJ (1997). PDGF-BB increases endothelial migration on cord movements during angiogenesis in vitro. *J Cell Biochem* **64** (3), 403-13.

Thompson EW, Nakamura S, Shima TB, Melchiori A, Martin GR, Salahuddin SZ, Gallo RC and Albini A (1991). Supernatants of acquired immunodeficiency syndrome-related Kaposi's sarcoma cells induce endothelial cell chemotaxis and invasiveness. *Cancer Res* **51** (10), 2670-6

Thompson HM and McNiven MA (2001). Dynamin: switch or pinchase? *Curr Biol* **11** (21), R850

Thornhill MH and Haskard DO (1990a). IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-gamma. *J Immunol* **145** (3), 865-72.

Thornhill MH, Kyan-Aung U and Haskard DO (1990b). IL-4 increases human endothelial cell adhesiveness for T cells but not for neutrophils. *J Immunol* **144** (8), 3060-5.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD and McDonald DM (1999). Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* **286** (5449), 2511-4

Tilghman RW and Hoover RL (2002). E-selectin and ICAM-1 are incorporated into detergent-insoluble membrane domains following clustering in endothelial cells. *FEBS Lett* **525** (1-3), 83-7

Tnani M and Bayard BA (1999). Evidence for IRF-1-dependent gene expression deficiency in interferon unresponsive HepG2 cells. *Biochim Biophys Acta* **1451** (1), 59-72.

Tooze SA, Martens GJ and Huttner WB (2001). Secretory granule biogenesis: rafting to the SNARE. *Trends Cell Biol* **11** (3), 116-22

Tracey KJ and Cerami A (1993). Tumor necrosis factor: an updated review of its biology. *Crit Care Med* **21** (10 Suppl), S415-22

Tripp CS, Wolf SF and Unanue ER (1993). Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc Natl Acad Sci U S A* **90** (8), 3725-9

Twigg HL, 3rd, Spain BA, Soliman DM, Knox K, Sidner RA, Schnizlein-Bick C, Wilkes DS and Iwamoto GK (1999). Production of interferon-gamma by lung lymphocytes in HIV-infected individuals. *Am J Physiol* **276** (2 Pt 1), L256-62

Uccini S, Ruco LP, Monardo F, Stoppacciaro A, Dejana E, La Parola IL, Cerimele D and Baroni CD (1994). Co-expression of endothelial cell and macrophage antigens in Kaposi's sarcoma cells. *J Pathol* **173** (1), 23-31

Ucer U, Bartsch H, Scheurich P, Berkovic D, Ertel C and Pfizenmaier K (1986). Quantitation and characterization of gamma-interferon receptors on human tumor cells. *Cancer Res* **46** (10), 5339-43.

Ullum H, Cozzi Lepri A, Bendtzen K, Victor J, Gotzsche PC, Phillips AN, Skinhoj P and Klarlund Pedersen B (1997). Low production of interferon gamma is related to disease progression in HIV infection: evidence from a cohort of 347 HIV-infected individuals. *AIDS Res Hum Retroviruses* **13** (12), 1039-46

van der Bliek AM (1999). Functional diversity in the dynamin family. *Trends Cell Biol* **9** (3), 96-102.

van Loon AP, Ozmen L, Fountoulakis M, Kania M, Haiker M and Garotta G (1991). High-affinity receptor for interferon-gamma (IFN-gamma), a ubiquitous protein occurring in different molecular forms on human cells: blood monocytes and eleven different cell lines have the same IFN-gamma receptor protein. *J Leukoc Biol* **49** (5), 462-73.

Vasse M, Pourtau J, Trochon V, Muraine M, Vannier JP, Lu H, Soria J and Soria C (1999). Oncostatin M induces angiogenesis in vitro and in vivo. *Arterioscler Thromb Vasc Biol* **19** (8), 1835-42.

Vestal DJ, Gorbacheva VY and Sen GC (2000). Different subcellular localizations for the related interferon-induced GTPases, MuGBP-1 and MuGBP-2: implications for different functions? *J Interferon Cytokine Res* **20** (11), 991-1000.

Vihinen P and Kahari VM (2002). Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. *Int J Cancer* **99** (2), 157-66

Volchuk A, Amherdt M, Ravazzola M, Brugger B, Rivera VM, Clackson T, Perrelet A, Sollner TH, Rothman JE and Orci L (2000). Megavesicles implicated in the rapid transport of intracisternal aggregates across the Golgi stack. *Cell* **102** (3), 335-48.

von Albertini MA, Stroka DM, Brostjan C, Stuhlmeier KM, Bach FH and Robson SC (1997). Adenosine nucleotides induce E-selectin expression in porcine endothelial cells. *Transplant Proc* **29** (1-2), 1062.

Vyakarnam A, Matear P, Meager A, Kelly G, Stanley B, Weller I and Beverley P (1991). Altered production of tumour necrosis factors alpha and beta and interferon gamma by HIV-infected individuals. *Clin Exp Immunol* **84** (1), 109-15

Wahbi A, Marcusson JA and Sundqvist KG (1996). Expression of adhesion molecules and their ligands in contact allergy. *Exp Dermatol* **5** (1), 12-9

Walker W, Aste-Amezaga M, Kastelein RA, Trinchieri G and Hunter CA (1999). IL-18 and CD28 use distinct molecular mechanisms to enhance NK cell production of IL-12-induced IFN-gamma. *J Immunol* **162** (10), 5894-901

Wanecek M, Weitzberg E, Rudehill A and Oldner A (2000). The endothelin system in septic and endotoxin shock. *Eur J Pharmacol* **407** (1-2), 1-15

Wang GX, Cai SX, Wang PQ, Ouyang KQ, Wang YL and Xu SR (2002). Shear-induced changes in endothelin-1 secretion of microvascular endothelial cells. *Microvasc Res* **63** (2), 209-17

Westermann R, Grothe C and Unsicker K (1990). Basic fibroblast growth factor (bFGF), a multifunctional growth factor for neuroectodermal cells. *J Cell Sci Suppl* **13** 97-117

Wilting J, Christ B, Bokeloh M and Weich HA (1993). In vivo effects of vascular endothelial growth factor on the chicken chorioallantoic membrane. *Cell Tissue Res* **274** (1), 163-72

Xerri L, Hassoun J, Planche J, Guigou V, Grob JJ, Parc P, Birnbaum D and deLapeyriere O (1991). Fibroblast growth factor gene expression in AIDS-Kaposi's sarcoma detected by in situ hybridization. *Am J Pathol* **138** (1), 9-15

Yamada Y, Nezu J, Shimane M and Hirata Y (1997). Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics* **42** (3), 483-8

Yamaji R, Adamik R, Takeda K, Togawa A, Pacheco-Rodriguez G, Ferrans VJ, Moss J and Vaughan M (2000). Identification and localization of two brefeldin A-inhibited guanine nucleotide-exchange proteins for ADP-ribosylation factors in a macromolecular complex. *Proc Natl Acad Sci U S A* **97** (6), 2567-72

Yang E, Wen Z, Haspel RL, Zhang JJ and Darnell JE, Jr. (1999). The linker domain of Stat1 is required for gamma interferon-driven transcription. *Mol Cell Biol* **19** (7), 5106-12.

Yawalkar N, Egli F, Hari Y, Nievergelt H, Braathen LR and Pichler WJ (2000). Infiltration of cytotoxic T cells in drug-induced cutaneous eruptions. *Clin Exp Allergy* **30** (6), 847-55

Yoon Y, Pitts KR, Dahan S and McNiven MA (1998). A novel dynamin-like protein associates with cytoplasmic vesicles and tubules of the endoplasmic reticulum in mammalian cells. *J Cell Biol* 140 (4), 779-93

Zanetta L, Marcus SG, Vasile J, Dobryansky M, Cohen H, Eng K, Shamamian P and Mignatti P (2000). Expression of Von Willebrand factor, an endothelial cell marker, is up-regulated by angiogenesis factors: a potential method for objective assessment of tumor angiogenesis. *Int J Cancer* 85 (2), 281-8

Zietz C, Hotz B, Stürzl M, Rauch E, Penning R and Lohrs U (1996). Aortic endothelium in HIV-1 infection: chronic injury, activation, and increased leukocyte adherence. *Am J Pathol* 149 (6), 1887-98

Annex:

HUVEC transduced with GBP-1 and ΔCAAX	From Kristin Töpolt, GSF, Munich
Monoclonal anti GBP-1 antibodies	In collaboration with Elisabeth Kremmer, GSF, Munich

Figure 1	From http://sprojects.mmi.mcgill.ca.htm
Figure 2	From http://atlases.muni.cz/atl_en/sect_main.html, with permission
Figure 3	From http://atlases.muni.cz/atl_en/sect_main.html, with permission
Figure 4	From http://atlases.muni.cz/atl_en/sect_main.html, with permission
Figure 5	From http://atlases.muni.cz/atl_en/sect_main.html, with permission
Figure 6	From http://atlases.muni.cz/atl_en/sect_main.html, with permission
Figure 7	Produced during this work
Figure 8	Produced during this work
Figure 9	Adapted from (Prakash, *et al*. 2000a)
Figure 10	Produced during this work
Figure 11	Produced during this work
Figure 12	Produced during this work
Figure 13	Produced during this work. Published in (Lubeseder-Martellato *et al*. 2002)
Figure 14	Produced during this work
Figure 15	Produced during this work
Figure 16	Produced during this work
Figure 17	Produced during this work. Published in (Guenzi *et al*. 2001)
Figure 18	Produced during this work
Figure 19	Produced during this work
Figure 20	Produced during this work. Published in (Guenzi *et al*. 2001)
Figure 21	Produced during this work. Published in (Guenzi *et al*. 2001)
Figure 22A	With the help of Anita Jörg GSF. Published in (Lubeseder-Martellato *et al*. 2002)
Figure 22B	Produced during this work
Figure 22C	Produced during this work
Figure 22D	Produced during this work with the help of Elisabeth Naschberger, GSF
Figure 23	Produced during this work. Published in (Lubeseder-Martellato *et al*. 2002)
Figure 24	Produced during this work
Figure 25	Produced during this work
Figure 26	Produced during this work
Figure 27	Produced during this work. Published in (Lubeseder-Martellato *et al*. 2002)
Figure 28	Produced during this work. Published in (Lubeseder-Martellato *et al*. 2002)
Figure 29	Produced during this work. Published in (Lubeseder-Martellato *et al*. 2002)
Figure 30	Produced during this work. Published in (Lubeseder-Martellato *et al*. 2002)
Figure 31	Produced during this work. Published in (Guenzi *et al*. 2001)
Figure 32	Produced during this work
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Figure 49	Produced during this work

The experimental parts of the work presented here were carried out in the laboratory of Prof. Dr. rer. nat. Michael Stürzl from August 1999 to December 2002 at Department of Virus-Induced Vasculopathy, Institute of Molecular Virology, GSF-National Research Center for Environment and Health, Neuherberg, Germany, D-85764.

Eidesstattliche Erklärung

Hiermit versichere ich, daß die vorliegende Arbeit von mir selbständig und unter ausschließlicher Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde.

Ferner erkläre ich, dass ich weder an der Universität München noch an einer anderen Universität versucht habe, eine Dissertation einzureichen.

München, 26 Mai 2003

OWN PUBLICATIONS

Parts of this thesis were published:

Lubeseder-Martellato C, Guenzi E, Jorg A, Topolt K, Naschberger E, Kremmer E, Zietz C, Tschachler E, Hutzler P, Schwemmle M, Matzen K, Grimm T, Ensoli B and Sturzl M (2002a). Guanylate-Binding Protein-1 Expression Is Selectively Induced by Inflammatory Cytokines and Is an Activation Marker of Endothelial Cells during Inflammatory Diseases. *Am J Pathol* **161** (5), 1749-59

Guenzi E, Topolt K, Cornali E, **Lubeseder-Martellato C** (*shared first autorship)*, Jorg A, Matzen K, Zietz C, Kremmer E, Nappi F, Schwemmle M, Hohenadl C, Barillari G, Tschachler E, Monini P, Ensoli B and Sturzl M (2001). The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *Embo J* **20** (20), 5568-77.

Patents submitted:

Lubeseder-Martellato C, Stürzl M, Kremmer E and Guenzi E (2002c). ELISA Verfahren zum Nachweis von Guanylate Bindung Protein-1(GBP-1). DE-Patentanmeldung Nr 102 60 265.4.

Other publications:

Stürzl M, **Lubeseder-Martellato C**, Töpolt K and Naschberger E (2003). Das Guanylat-Bindungsprotein-1: ein neuer Marker und Regulator der Gefäßaktivierung bei Entzündungen mit Perspektiven für die antiangiogene Tumortherapie. *Biofourm* **3**, 122-24.

Guenzi E, Topolt K, **Lubeseder-Martellato C**, Jorg A, Naschberger E, Benelli R, Albini A and Sturzl M (2003). The guanylate binding protein-1 GTPase controls the invasive and angiogenic capability of endothelial cells through inhibition of MMP-1 expression. *J* **22** (15), 3772-3782.

Naschberger E, Werner T, Vicente A, Guenzi E, **Lubeseder-Martellato C**, Topolt K, Nelson P and Sturzl M. A cRel/ISRE promotor module triggers inflammatory cytokine-induced GBP-1 expression in endothelial cells. (*in revision*)

Posters and congress-proceedings:

Stürzl M, **Lubeseder-Martellato C**, Töpolt K, Jörg A, Naschberger E, Kremmer E, Zietz C and Guenzi E. The guanylate-binding protein-1 characterizes and regulates the inflammatory cytokine-actived phenotype of endothelial cells. September 21-24, (2002). 2nd International Symposium of the German Priority Research Program Angiogenesis. Kloster Seeon, Germany

Lubeseder-Martellato C, Guenzi E, Töpolt K, Jörg A, Naschberger E, Kremmer E and Stürzl M. The Human Guanylate Binding Protein-1 is Localized in Membranous Vesicles in Endothelial Cells Activated by Inflammatory Cytokines .Exocytosis; membrane structure and dynamics. April 20 - 25 (2002), (2002b). Joint EURESCO Conference / EMBO workshop. Tomar, Portugal

Martellato C, Guenzi E, Töpolt K, Jörg A, Quadt I, Kremmer E, Tschachler E, Zietz C, Grimm T and Stürzl M. The Guanylate Binding Protein-1: a Novel Marker of Endothelial Cell Activation by Inflammatory Cytokines in vivo. May 24-26, (2001). III Symposium on the biology of endohtelial cells. Giessen, Germany

Stürzl M, Cornali E, **Martellato C**, Töpolt K, Jörg A, Matzen K, Nappi F, Schwemmle M, Zietz C, Monini P, Ensoli B and Guenzi E. The inflammatory cytokine-induced inhibition of endothelial cell proliferation is mediated by the guanylate binding protein-1. May 24-26, (2001). III Symposium on the biology of endohtelial cells. Giessen, Germany.

Töpolt K, Cornali E, **Martellato C**, Jörg A, Ensoli B, Guenzi E and Stürzl M. Structure-function relationship of the human guanylate binding protein-1 and its antiproliferative activity in endothelial cells. May 24-26 . (2001). III Symposium on the biology of endohtelial cells. Giessen, Germany

Grimm T, Guenzi E, **Martellato C**, Jörg A, Ensoli B, Guenzi E and Stürzl M. Human herpes virus-8 (HHV-8) anti-apoptotic factors regulate progression of Kaposis's sarcoma. May 24-26, (2001). III Symposium on the biology of endohtelial cells. Giessen, Germany

Stürzl M, Cornali E, **Martellato C**, Töpolt K, Jörg A, Matzen K, Nappi F, Schwemmle M, Zietz C, Monini P, Ensoli B and Guenzi E. The human guanylate binding protein-1 is a novel inhibitor of endothelial cell proliferation. April 24-29, (2001). Keystone symposia, angiogenesis and chronic disease. Keystone, USA

Guenzi E, Cornali E, **Martellato C**, Zietz C, Nappi F, Monini P, Schwemmle M, Ensoli B and Stürzl M (2000). Novel approaches for determination of endothelial cell activation in the pathogenesis of Kaposis's sarcoma. *I congress on viral diseases.* Infection, **28, supp. 1** Munich, Germany

Stürzl M, Guenzi E, **Martellato C**, Matzen K, Töpolt K, Jörg A, Li B, Stelz G, Zietz C, Martellato C and Ensoli B (2000). Human herpes virus-8 (HHV-8) gene expression in Kaposi´s sarcoma primary lesions. *I congress on viral diseases.* Infection, **28, supp. 1,** Munich, Germany

Guenzi E, Cornali E, **Martellato C**, Zietz C, Nappi F, Monini P, Schwemmle M, Ensoli B and Stürzl M. The human guanylate binding protein-1 (GBP)-1: a novel regulator of endothelial cell activation in the development of Kaposis's sarcoma. October 19-21, (2000). Hersonissos, Creta

Guenzi E, Cornali E, **Martellato C**, Zietz C, Nappi F, Monini P, Schwemmle M, Ensoli B and Stürzl M. The human guanylate binding protein-1 (GBP)-1: a novel regulator of endothelial cell activation in the development of Kaposis's sarcoma. August 31- September 1, (2000). II international conference of the Mor Kaposa Foundation. Budapest, Hungary

Presentations:

Lubeseder-Martellato C, Guenzi E, Töpolt K, Jörg A, Naschberger E, Tschachler E, Brockmeyer N and Stürzl M. The guanylate binding protein-1 is a tissue and serum marker of inflammatory cytokine-activated endothelial cells. Juli 18-20, (2003). IV Symposium on the biology of endothelial cells. Munich, Germany

CURRICULUM VITAE

Personal data

Family name:	Lubeseder-Martellato
First name:	Clara
Date of birth:	September, 25th, 1974
Place of birth:	Venice, Italy
Marital status:	Married
Private address:	Rümannstr. 41, 80804-Munich, Germany

Education

1980 - 1988	Primary School in Mestre (VE), Italy
1988 - 1993	Secondary School in Mestre (VE), at the scientific lyceum "Morin""

Academic education and scientific experience

1993 - 1998	Studies of Biology at the Faculty of Mathematics and Natural Sciences, University of Padua, Italy.
1996 - 1997	Erasmus scholarship at the LMU, Munich, Germany
November 1998	Degree in Molecular Biology. Experimental thesis: "Establishment of a method for a DSCA screening of the whole coding region of the dystrofin gene" in the laboratory of Prof. Dr. GA Danieli, University of Padua, Italy.
1998 - 1999	Training in the laboratory of Prof. Dr. GA Danieli, University of Padua.
1999 - 2002	Ph.D. study in the department of Virus-induced Vasculopathy at the Institute of Molecular Virology at the National Research Center for Environment and Health (GSF), Munich, Germany, under the supervision of Prof. Dr. Michael Stürzl. Topic of the research: "The guanylate-binding-protein-1: a molecular marker of the inflammatory cytokine-activated phenotype of endothelial cells."
Mai 2001	Participation at the 3rd Symposium of the Biology of Endothelial Cells. "Pathophysiology of the endothelium: vascular and infectious diseases", Giessen, Germany.
April 2002	Participation at the joint EMBO/EURESCO conference "Exocytosis, membrane structure and dynamics", Tomar, Portugal.
Februar 2002	Participation at the course for "Erhaltung der Sachkunde nach den §§ 15 und 17 GenTSV für Projektleiter und Beauftragte der Biologische Sicherheit"

Prices

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