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Human guanylate binding protein-1 (hGBP-1) characterizes and establishes a non-angiogenic endothelial cell activation phenotype in inflammatory diseases

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Introduction

Mammalian cells require oxygen and nutrients for their survival. Therefore, all cells are located within a distance below 100–200 μm (the diffusion limit of oxygen) to blood vessels. Organ and tissue growth beyond this size require recruitment of new blood vessels by vasculogenesis or angiogenesis. Both processes are strictly regulated by pro- and anti-angiogenic molecules. The activities of both groups of factors are in equilibrium in normal tissues, whereas a switch towards the angiogenic or anti-angiogenic side is observed in various diseases (Carmeliet, 2000; Carmeliet and Jain, 2000).

Endothelial cells (EC) form the inside of blood vessels, constituting the endothelium. The endothelium is among the largest "organs" of the body. The surface of the endothelium in an adult human covers more than 1000 m^2, is composed of more than 10^{12} cells and weighs approximately 1 kg (Augustin et al., 1994; Cines et al., 1998).

The blood vessel endothelium plays an important role in inflammatory processes and among other functions provides an adhesive surface for leukocyte extravasation

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Table 1
Opposite activities of inflammatory cytokines (IFN, IL-1 and TNF-α) and angiogenic growth factors (VEGF and bFGF) in endothelial cells. Inhibition indicated by (↓), induction by (↑)

Angiogenic activities	Inflammatory cytokines	Angiogenic growth factors	References
Proliferation	↓	↑	Frater-Schroder et al. (1987), Guenzi et al. (2001), Yilmaz et al. (1998), Ferrara (2004)
Invasion	↓[a]	↑	Guenzi et al. (2003), Detmar et al. (2000)
Adhesiveness for leukocytes	↑	↓	Zhang and Frei (2001), Dirkx et al. (2003), Thornhill et al. (1991), Tromp et al. (2000), Griffioen et al. (1999)
MMP-1 expression	↓	↑	Ala-aho et al. (2000), Guenzi et al. (2003), Wary et al. (2003), Sato et al. (2000)

[a]Invasion was determined in collagen-I matrices.

into the tissues (Tan et al., 1999). To this goal it switches from a non-activated to an activated state (Pober et al., 1986). This switch is the net result of an integrated dose- and time-dependent cellular response to various stimuli originating from the blood or neighboring cells (Tan et al., 1999). The most potent and best characterized modulators of endothelial cell activation are inflammatory cytokines (IC), such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ, and angiogenic growth factors (AGF), such as vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Pober and Cotran, 1990; Ferrara, 2004).

IC and AGF have opposite effects on ECs (Table 1). IC inhibit EC proliferation (Frater-Schroder et al., 1987; Yilmaz et al., 1998; Guenzi et al., 2001) and invasiveness in collagen-I matrices (Guenzi et al., 2003), but induce adhesiveness of EC for leukocytes (Thornhill et al., 1991; Zhang and Frei, 2001; Dirkx et al., 2003). In contrast, AGF increase EC proliferation (Ferrara, 2004) and invasiveness (Detmar et al., 2000), e.g. by activating the expression of matrix metalloproteinases (MMPs) (Sato et al., 2000; Wary et al., 2003), but inhibit EC adhesiveness for leukocytes (Griffioen et al., 1999; Tromp et al., 2000). In consequence, IC and AGF promote opposite activation phenotypes of EC. IC induce an adhesion-competent, non-angiogenic phenotype, whereas AGF lead to an angiogenic phenotype with a reduced adhesiveness for leukocytes. Both extravasation of leukocytes and angiogenesis are key processes in inflammatory tissues (Tan et al., 1999). Therefore, the appearance of the IC- and AGF-induced activation phenotypes of EC has to be coordinated in a time- and space-dependent manner.

Molecular markers of inflammatory vessel activation

Efforts to detect distinct activation phenotypes of EC in vivo used different approaches. Antibodies that specifically recognize a complex of an activating factor

with its endothelial cell surface receptor were used. For example, a conformational epitope at the N-terminus of VEGF-A that is created upon binding of VEGF-A to its receptor is recognized by the monoclonal antibody 11B5 (Koukourakis et al., 2000). Using this antibody VEGF-A-activated EC were identified in human tissues and it was shown that the number of VEGF-A-activated EC was directly related to the vessel number and angiogenic activity in human tumors (Koukourakis et al., 2000).

Another approach to detect different EC activation phenotypes in vivo is to determine the expression of specific marker proteins. By this approach an EC activation can be analyzed not only immunohistochemically in tissues at the single cell level, but also by serological means in case the EC release the marker protein into the blood. For instance, soluble intercellular adhesion molecule (sICAM), Thy-1, von Willebrand factor and thrombospondin are released by activated EC. In the sera of patients with inflammation and sepsis these proteins are present in increased concentrations (Tannenbaum and Gralnick, 1990; Kayal et al., 1998; Saalbach et al., 1999). Unfortunately, none of these proteins are specifically expressed by EC, or allow to differentiate between IC- and AGF-activated EC. sICAM is not exclusively released from IC-stimulated EC, but also from keratinocytes, fibroblasts and epithelial cells (Hashimoto et al., 1994; Leung, 1999). Similarly, thy-1 is secreted from EC and fibroblasts (Saalbach et al., 1999). The release of von Willebrand factor is increased by IL-1β (Giddings and Shall, 1987), but inhibited by IFN-γ and TNF-α (Tannenbaum and Gralnick, 1990). The secretion of thrombospondin is inhibited by IL-1β and TNF-α but not affected by IFN-γ (Morandi et al., 1994). Also for immunohistochemical studies no specific markers are available to differentiate between the IC- and the AGF-activated phenotypes of EC.

We aimed to identify a marker gene that is selectively expressed in IC-activated endothelial cells. Our screening retrieved human guanylate binding protein-1 (hGBP-1), a protein that was found to be specifically induced in endothelial cells by IC such as IFN-α/γ, IL-1α/β and TNF-α in vivo (Guenzi et al., 2001; Lubeseder-Martellato et al., 2002). Angiogenic growth factors (VEGF, bFGF) alone did not alter hGBP-1 expression in EC and repressed the stimulation of hGBP-1 expression by IC (Lubeseder-Martellato et al., 2002). These results suggested that hGBP-1 expression may characterize EC in a microenvironment that is dominated by IC.

The guanylate binding proteins: a subfamily of the large GTPases

GBPs were originally identified as the most abundant proteins in extracts of human fibroblasts treated with interferons, IFN-γ being the most effective (Cheng et al., 1983). Presently, five different GBPs (GBP-1 to GBP-5) with molecular weights of 65–71 kDa are known, both in man and mouse. The GBPs establish a subfamily within the protein family of large GTPases (Fig. 1). This protein family includes the atlastins (Zhu et al., 2003), the Mx proteins (Aebi et al., 1989) and dynamin (Diatloff-Zito et al., 1995), although the overall primary sequence homology is low. The relationship of these proteins is more evident at the structural level, and by

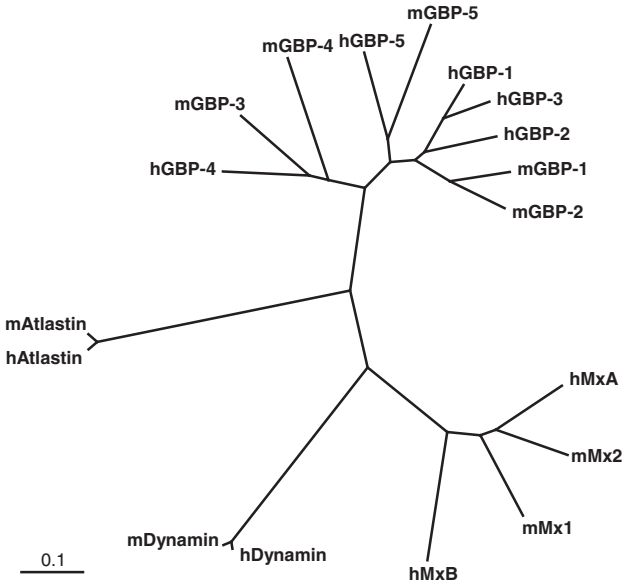

Fig. 1. Phylogram of human and murine large GTPases. GBPs establish a protein subfamily within the large GTPases. Amino acid sequences of hGBP-1 (accession number M55542), hGBP-2 (BC073163), hGBP-3 (NM_018284), hGBP-4 (AF288814), hGBP-5 (AF288815), hAtlastin (NM_015915), hMxA (NM_002462), hMxB (M30818), hDynamin (NM_004408), and respective mouse proteins [accession numbers of mGBPs as in (Nguyen et al., 2002)] mAtlastin (NM_178628), mMx1 (BC011113), mMx2 (NM_013606), and mDynamin (NM_010065) were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/) at the European Bioinformatics Institute.

shared biochemical features like nucleotide-dependent oligomerization and high turnover GTPase activity (Prakash et al., 2000b). Alignment of the amino acid sequences of all five GBPs of mouse and man features substantial primary sequence homology in the N-terminal parts that harbor the GTPase activity and lower homology in the C-terminal parts of the proteins (Nguyen et al., 2002). Murine GBP-1 (mGBP-1) and -2 (mGBP-2) reveal 80% identical amino acids, hGBP-1 and -2 75% and hGBP-1 and mGBP-2 68% (Nguyen et al., 2002). However, GBPs reveal also different features. For example, hGBP-1 and mGBP-2 display a granular cytoplasmic expression and are partially localized in vesicle-like structures, whereas mGBP-1 shows a diffuse homogeneous cytoplasmic expression and cannot be detected in vesicles (Vestal et al., 2000; Gorbacheva et al., 2002; Lubeseder-Martellato et al., 2002). These results suggest that the members of the GBP family may reveal overlapping as well as distinct features.

Structure and biochemical features of guanylate binding proteins

All GBPs bind a complex of guanine nucleotides and Mg^{2+} with relative low affinity (Prakash et al., 2000b). Typical GTPase domains harbor three classical

GTP-binding motifs: the phosphate-binding P-loop GXXXXGK(S/T), the phosphate- and Mg^{2+}-binding DXXG motif (G, glycine; K, lysine; S, serine; D, aspartic acid; T, threonine; and X, any amino acid) and the guanine nucleotide-specificity providing (N/T)KXD motif [N, asparagine; (Dever et al., 1987)]. In human and murine GBPs the classical (N/T)KXD motif is substituted by a conserved arginine–aspartic acid (RD)-motif (Praefcke et al., 1999; Prakash et al., 2000b).

hGBP-1 is the best-characterized member of the GBP family. It hydrolyzes GTP with a high intrinsic rate of GTP hydrolysis ($k_{cat} = 2-100\,min^{-1}$) (Schwemmle and Staeheli, 1994; Prakash et al., 2000a). In contrast to other GTPases, hGBP-1 hydrolyzes GTP mainly to GMP and inorganic phosphate. GDP is an intermediate reaction product, but is released as side product only at suboptimal reaction conditions (Schwemmle and Staeheli, 1994). Although hGBP-1 can bind GDP, GDP from bulk solution never serves as substrate for subsequent cleavage to GMP (Schwemmle and Staeheli, 1994; Praefcke et al., 1999). The GTPase of hGBP-1 works independent of guanine nucleotide exchange factors or GTPase activating proteins (GAP) (Prakash et al., 2000b). hGBP-1 like dynamin and Mx-proteins forms oligomers upon binding of guanine nucleotides and oligomer formation largely increases the specific hydrolytic activity (Schwemmle et al., 1995; Warnock et al., 1996; Prakash et al., 2000a).

The crystal structure of hGBP-1 has been resolved both in the GTP-bound and in the nucleotide-free state (Prakash et al., 2000a,b). Both structures were found to be very similar (Prakash et al., 2000a, b). The hGBP-1 structure exhibits two domains: (1) a N-terminal compact globular domain that contains the GTPase function (Fig. 4, pink), and (2) a long, index finger-like purely α-helical C-terminal domain (Fig. 4, green). The DXXG motif (residues 97–100) of hGBP-1 superimposes well with the respective motif of the small GTPase ras. In contrast, the P-loop of hGBP-1 (residues 45–52) and the RD-motif (residues 183–184) are located at different positions. Also, the guanine base of GTP is localized in a different position relative to its ribose moiety in hGBP-1 as compared to ras. Unique structural motifs of hGBP-1 are the guanine and the phosphate caps (residues 251–258, resp. 65–76). These motifs form a hydrophobic binding pocket for the guanine base and shield GTP from the solvent, respectively. These features indicate that GTP hydrolysis by GBPs may follow a distinct mechanism (Prakash et al., 2000b).

hGBP-1 expression in vitro and in vivo

In vitro IFN-γ induces hGBP-1 expression in many different cell types, including endothelial cells, fibroblasts, keratinocytes, B-cells, T-cells and peripheral blood mononuclear cells (Guenzi et al., 2001, 2003; Lubeseder-Martellato et al., 2002). We showed that hGBP-1 expression in EC is not only induced by IFN-γ but also by IFN-α, TNF-α and IL-1α/β (Guenzi et al., 2001; Lubeseder-Martellato et al., 2002). These factors selectively induced hGBP-1 expression in EC, whereas other cytokines (IL-4, IL-6, IL-10, IL-18, oncostatin M), chemokines (macrophage chemoattractant protein-1, macrophage inflammatory protein-1β, platelet factor 4, IFN-γ-inducible

protein-10, stromal derived factor-1α) and growth factors (bFGF, VEGF, angiopoietin-2, platelet-derived growth factor B/B) did not (Guenzi et al., 2001; Lubeseder-Martellato et al., 2002). Of note, all of the IC that induce hGBP-1 expression inhibit EC proliferation and promote cell adhesiveness for leukocytes (Frater-Schroder et al., 1987; Thornhill et al., 1991; Yilmaz et al., 1998; Guenzi et al., 2001; Zhang and Frei, 2001).

Interestingly, AGF repressed IC-induced hGBP-1 expression in a dose-dependent manner. Vice versa, increasing concentrations of IC up-regulated hGBP-1 expression in EC also in the presence of AGF (Guenzi et al., 2001). Most importantly, at combined application of IC and AGF the level of hGBP-1 expression was directly related to the manifestation of IC-associated cell biologic effects in EC such as the inhibition of proliferation, inhibition of MMP-1 expression and inhibition of invasiveness into collagen-I matrices (Guenzi et al., 2001, 2003). Altogether, these in vitro results indicated that hGBP-1 characterizes EC that are predominantly exposed to IC.

In order to investigate whether hGBP-1 may also characterize IC-activated endothelial cells in vivo, we developed a monoclonal antibody (1B1) to detect hGBP-1 in paraffin-embedded tissue sections (Guenzi et al., 2001; Lubeseder-Martellato et al., 2002). hGBP-1 expression was investigated in normal skin and different skin diseases with a high inflammatory component, such as adverse drug reaction, psoriasis and Kaposi's sarcoma (Stürzl et al., 2001). All inflammatory skin diseases displayed high hGBP-1 expression, whereas it was undetectable in normal skin (Fig. 2) (Lubeseder-Martellato et al., 2002).

In contrast to the in vitro situation hGBP-1 was highly associated with blood vessel endothelial cells and only rarely observed in other cells (Lubeseder-Martellato et al., 2002). hGBP-1 expression in inflammatory tissues was restricted to single blood vessels that were surrounded by numerous monocytes which expressed high concentrations of IC (Guenzi et al., 2001; Lubeseder-Martellato et al., 2002). Endothelial cells of vessels that were located in tissue areas rich in angiogenic growth factors did not stain for hGBP-1 (Guenzi et al., 2001; Lubeseder-Martellato et al., 2002). The expression pattern of hGBP-1 in vivo demonstrated that this protein will be a useful marker to map the temporal and spatial appearance of IC-activated EC in inflammatory diseases.

Transcriptional regulation of hGBP-1

At present only few genes (e.g. ICAM-1) have been described, whose expression is induced by IFN, IL-1 and TNF-α (Dustin et al., 1986; Pober et al., 1987).

Three different transcription factor binding sites are involved in IC-mediated activation of hGBP-1 expression: an interferon-α response element (ISRE), a γ-interferon activation site (GAS) and a cRel binding site (Lew et al., 1991; Naschberger et al., 2004). The latter transcription factor binding site is activated by proteins belonging to the NF-κB family that is comprised of five proteins p65, p50, cRel, RelB and p52 (Pahl, 1999).

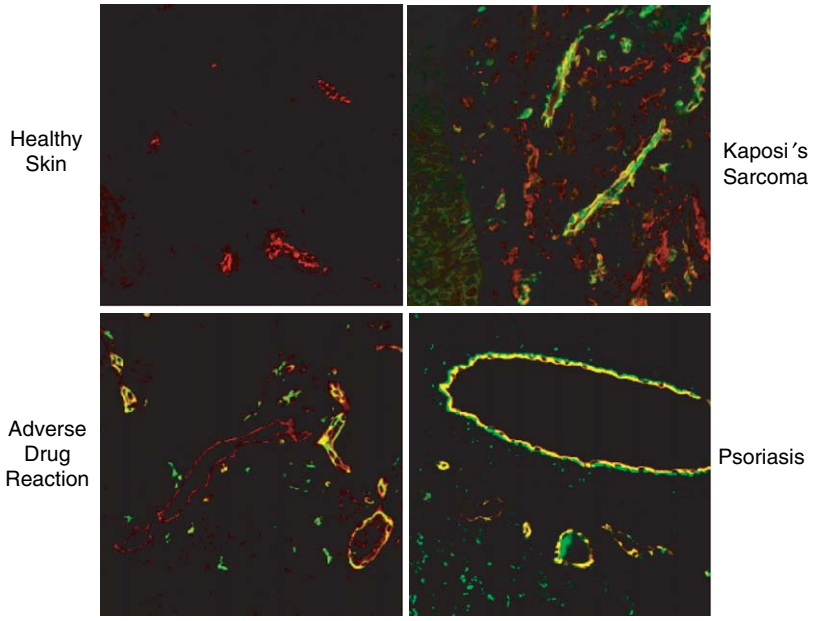

Fig. 2. Expression of hGBP-1 in inflammatory tissues. Immunofluorescence detection of hGBP-1 in healthy skin and different skin diseases with an inflammatory component (psoriasis, adverse drug reaction and Kaposi's sarcoma). The endothelial cell marker CD31 was stained in red, hGBP-1 in green. Yellow color indicates colocalization [Reprinted from Am J Pathol 2002;161:1749–1759 with permission from the American Society for Investigative Pathology].

IFN-α and IFN-γ activate hGBP-1 expression via ISRE and GAS, but engage different signaling molecules (Decker et al., 1989; Lew et al., 1989). IFN-α activates interferon stimulated gene factor-3 (ISGF3) (Levy et al., 1989) and IFN-α activation factor (AAF). Activated ISGF3 binds to ISRE and AAF to GAS of the hGBP-1 promoter (Decker et al., 1991a). IFN-γ activates interferon regulatory factor-1 (IRF-1) (Kroger et al., 2002) and STAT1 (Darnell, 1997). Activated IRF-1 binds to ISRE (Kroger et al., 2002). Activated STAT1 homodimerizes and the dimers are designated γ-IFN activation factor (GAF) (Decker et al., 1991b). GAF translocates into the nucleus and binds to GAS (Darnell, 1997). Of note, GAF binding mediates the early transcriptional response (<1 h) to IFN-γ, whereas IRF-1 regulates hGBP-1 transcription in the later phases of activation (>1 h) (Decker et al., 1991b; Mirkovitch et al., 1992).

IL-1β and TNF-α activate hGBP-1 expression via IRF-1 and NF-κB (p65/p50) that bind to ISRE and the cRel motif, respectively (Naschberger et al., 2004). Both binding sites cooperate in the induction of hGBP-1 transcription. Interestingly, ectopic expression of NF-κB isoforms and the use of isoform-specific inhibitors showed that NF-κB p50, rather than p65 activates the hGBP-1 promoter (Naschberger et al., 2004). The same unusual response to the NF-κB isoforms has also been observed in the transcriptional activation of another inflammation-associated protein, the C-reactive protein (CRP) (Clyne and Olshaker,

1999; Cha-Molstad et al., 2000; Agrawal et al., 2001). Preliminary results of our laboratory indicate that the promoter elements involved in hGBP-1 transcription are more sensitive for IC-activation in EC as compared to other cell types (Naschberger, unpublished observation), which may explain the EC-specific hGBP-1 expression in vivo.

Functions of hGBP-1

In analogy to Mx proteins (Haller et al., 1979; Horisberger et al., 1983) ectopic expression of hGBP-1 has been reported to induce an antiviral effect against vesicular stomatitis virus and encephalomyocarditis virus in HeLa cells (Anderson et al., 1999). Recently we have shown that hGBP-1, in addition, mediates two important anti-angiogenic effects of IC in EC (Guenzi et al., 2001, 2003).

First, hGBP-1 regulates the anti-proliferative effect of IC (Fig. 4) (Guenzi et al., 2001). hGBP-1 anti-proliferative activity occurred in the absence of apoptosis and was found to be independent of the GTPase activity and isoprenylation. Structure–function analysis showed that the C-terminal helical domain of hGBP-1 is necessary and sufficient for the inhibition of EC proliferation (Guenzi et al., 2001; Töpolt et al., 2002).

Second, hGBP-1 mediates the inhibition of EC invasiveness by IC via down-regulation of MMP-1 expression (Guenzi et al., 2003). MMP-1 is an important proteolytic enzyme in tissue remodelling (Guenzi et al., 2003). In contrast to the anti-proliferative effect of hGBP-1 its GTPase activity was necessary for the inhibition of MMP-1 expression.

Inhibition of MMP-1 expression by hGBP-1 abrogated the invasive and tube-forming capability of ECs in three-dimensional collagen-I matrices (Fig. 3) (Guenzi et al., 2003). Ectopic expression of a GTPase-deficient mutant hGBP-1 (D184N-hGBP-1: exchange of one amino acid in the active center of the GTPase) inhibited endogenous hGBP-1 activity in a transdominant negative manner and thereby, reconstituted MMP-1 expression and tube-forming capability of IC-treated endothelial cells [Fig. 3, (Guenzi et al., 2003)]. In addition, supplementation with exogenous MMP-1 antagonized the effect of endogenous hGBP-1 and reconstituted the tube-forming capability of endothelial cells in collagen-I matrices (Guenzi et al., 2003).

Of note, IC-induced adhesiveness of EC for leukocytes was not affected by hGBP-1 (Guenzi et al., 2001). Thus, hGBP-1 selectively mediates the anti-angiogenic effects of IC and may provide a molecular target to dissect the complex activity of IC on EC (Fig. 4). The anti-angiogenic activity of hGBP-1 involves two different parts of the molecule. The helical part of hGBP-1 is sufficient for the inhibition of EC proliferation, whereas the GTPase function is additionally required for the abrogation of MMP-1 expression and cell invasiveness (Fig. 4).

Most importantly, immunohistochemical stainings of inflammatory tissues showed that hGBP-1 expression in blood vessel EC is inversely related to both cell proliferation and MMP-1 expression (Guenzi et al., 2001, 2003). Thus, in vivo hGBP-1 characterizes and regulates the manifestation of a non-angiogenic

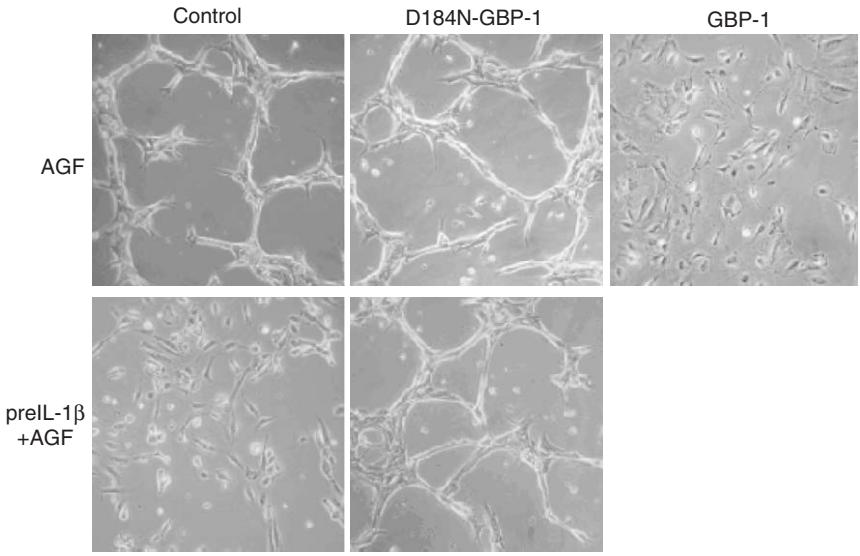

Fig. 3. hGBP-1 inhibits tube-forming capability of EC in three-dimensional collagen-I matrices via downregulation of MMP-1 expression. Tube formation in 3D collagen-I matrices of HUVEC that were transduced with the retroviral control vector (control) or the respective vectors that constitutively express a GTPase-deficient hGBP-1 (D184N-hGBP-1) or wild type hGBP-1 (GBP-1). HUVEC were either treated solely with AGF (10 ng/ml; AGF = VEGF and bFGF) for 48 h (upper panel) or in addition pretreated with IL-1β (200 U/ml) for 24 h (preIL-1β + AGF; lower panel) and were then grown in 3D collagen-I matrices with AGF for 48 h [modified with permission from (Guenzi et al., 2003)].

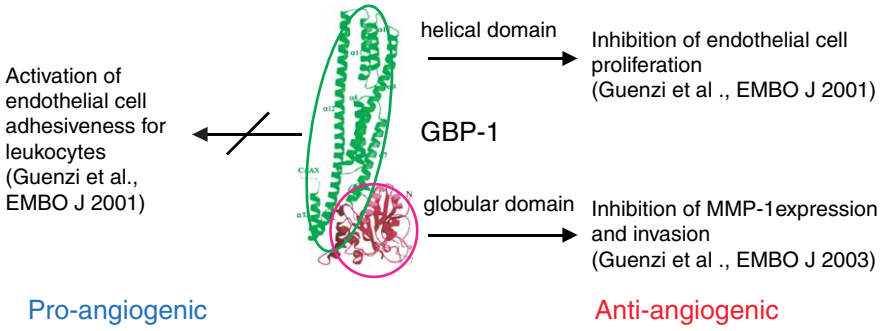

Fig. 4. hGBP-1 selectively mediates the anti-angiogenic effects of inflammatory cytokines in endothelial cells. The guanylate binding protein-1 can be divided into two domains of approximately the same size: a compact globular domain carrying the GTPase activity at the N-terminus (pink) and a long helical domain comprised of α-helices at the C-terminus (green). The nucleotide binding area is indicated by a sphere. hGBP-1 does not affect EC adhesiveness for leukocytes, but mediates the anti-angiogenic activities of EC. The helical domain is sufficient to inhibit EC proliferation. The GTPase activity of hGBP-1 in EC is required for the inhibition of MMP-1 expression and cell invasiveness [Reprinted from Prakash et al., 2000a with permission from the Nature Publishing Group; http://www.nature.com/nature/index.html <http://www.nature.com/nature/index.html>].

IC-induced EC phenotype that clearly can be differentiated from the AGF-induced phenotype of EC. hGBP-1 may be an useful tool to map the IC-induced activation phenotype of EC in inflammation and a target to modulate blood vessel growth in ischemia and tumor angiogenesis.

Summary

Blood vessel activation in inflammatory diseases is triggered by a myriad of different factors that partially reveal opposite activities on endothelial cells (EC). For example, inflammatory cytokines (IC) inhibit EC proliferation and induce cell adhesiveness for leukocytes. In contrast, angiogenic growth factors (AGF) activate EC proliferation and inhibit cell adhesiveness for leukocytes. In consequence, IC and AGF may induce two different activation phenotypes in EC that appear in a temporally and/or spatially coordinated manner in inflammatory tissues. Human guanylate binding protein-1 (hGBP-1) is a member of the large GTPase protein family. New results demonstrate that hGBP-1 is a specific marker of IC-activated EC that allows to differentiate the IC- and AGF-activated phenotype of EC at the single cell level, both in vitro and in vivo. In addition, hGBP-1 is the key mediator of the inhibitory effects of IC on EC proliferation and invasiveness. Both the expression pattern of hGBP-1 and its activity in EC supported the hypothesis that IC- and AGF-activation induce distinct adversely related phenotypes in EC. In future, hGBP-1 may be used as a marker to monitor the IC-induced phenotype of EC in inflammation and may also be exploited as a target to modulate EC activity in inflammatory diseases and tumor angiogenesis.

Acknowledgments

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