American Journal of Pathology, Vol. 161, No. 5, November 2002

Guanylate-Binding Protein-1 Expression Is Selectively Induced by Inflammatory Cytokines and Is an Activation Marker of Endothelial Cells during Inflammatory Diseases

Clara Lubeseder-Martellato,* Eric Guenzi,* Anita Jörg,* Kristin Töpolt,* Elisabeth Naschberger,* Elisabeth Kremmer,† Christian Zietz,‡ Erwin Tschachler,§ Peter Hutzler,¶ Martin Schwemmle,‖ Kathrin Matzen,* Thomas Grimm,* Barbara Ensoli,** and Michael Stürzl*

From the Department of Virus-Induced Vasculopathy, Institute of Molecular Virology, and the Department of Pathology,¶ GSF-National Research Center for Environment and Health, Neuherberg, Germany; the Institute of Molecular Immunology,† GSF-National Research Center for Environment and Health, Munich, Germany; the Institute of Pathology,‡ Ludwig Maximilians University, Munich, Germany; the Department of Virology,‖ Institute of Medical Microbiology and Hygiene, University of Freiburg, Freiburg, Germany; the Department of Dermatology,§ Division of Immunology, Allergy, and Infectious Diseases, University of Vienna Medical School, Vienna, Austria; and the Laboratory of Virology,** Retrovirus Division, Istituto Superiore di Sanità, Rome, Italy*

During angiogenesis and inflammatory processes, endothelial cells acquire different activation phenotypes, whose identification may help in understanding the complex network of angiogenic and inflammatory interactions *in vivo*. To this goal we investigated the expression of the human guanylate-binding protein (GBP)-1 that is highly induced by inflammatory cytokines (ICs) and, therefore, may characterize IC-activated cells. Using a new rat monoclonal antibody raised against GBP-1, we show that GBP-1 is a cytoplasmic protein and that its expression in endothelial cells is selectively induced by interferon-γ, interleukin-1α, interleukin-1β, or tumor necrosis factor-α, but not by other cytokines, chemokines, or growth factors. Moreover, we found that GBP-1 expression is highly associated with vascular endothelial cells as confirmed by the simultaneous detection of GBP-1 and the endothelial cell-associated marker CD31 in a broad range of human tissues. Notably, GBP-1 expression was undetectable in the skin, but it was highly induced in vessels of skin diseases with a high-inflammatory component including psoriasis, adverse drug reactions, and Kaposi's sarcoma. These results indicate that GBP-1 is a novel cellular activation marker that characterizes the IC-activated phenotype of endothelial cells. *(Am J Pathol 2002, 161:1749–1759)*

The healthy luminal endothelium consists of quiescent endothelial cells that provide a regularly organized and non-adhesive surface toward the blood constituents.[1] However, this interface is dynamic and, under appropriate stimulation, endothelial cells can undergo profound changes leading to an activated phenotype.[2] Activation of endothelial cells is implicated in numerous physiological functions and pathological dysfunctions of the vasculature, especially during development, inflammatory processes,[3] atherosclerosis,[4,5] and tumor angiogenesis.[6–8]

The activated endothelial cell phenotype represents a time- and dose-integrated response to various stimuli originating from the blood and/or from neighboring cells and tissues. Many soluble mediators including cytokines, chemokines, and growth factors are involved in the regulation of a restricted number of endothelial cell activities such as proliferation, adhesiveness, apoptosis, and chemotaxis. This suggests that different factors may have redundant and/or overlapping activities. In particular, angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induce endothelial cell proliferation,[9–12] whereas inflammatory cytokines (ICs) such as interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ induce endothelial cell adhesiveness for mono-

Supported by grants from the BioFuture program of the Bundesministerium für Bildung und Forschung, the Deutsche Krebshilfe, the Government of Bavaria (Bavaria-Quebec Research Co-operation) (to M. S.), the Associazione Italiana per la Ricerca sul Cancro, and the IX AIDS project from the Ministry of Health (to B. E.).

C. L.-M. and E. G. contributed equally to this work.

Accepted for publication August 2, 2002.

Address reprint requests Priv. Doz. Dr. rer. nat. Michael Stürzl, Department of Virus-Induced Vasculopathy, Institute of Molecular Virology, GSF-National Research Center for Environment and Health, Ingolstädter Landstraβe 1, D-85764 Neuherberg, Germany. E-mail: stuerzl@gsf.de.

cytes.[13,14] Focusing on the activation phenotypes of endothelial cells may help in elucidating the complex network of cellular events present during angiogenesis and inflammatory processes.

The guanylate-binding proteins (GBP)-1 and -2 are the most abundant proteins induced by IFN-γ.[15,16] GBPs have a concentration-dependent intrinsic high-turnover GTPase activity.[17–19] In particular, based on the crystal structure of GBP-1[20,21] and on biochemical considerations, it was proposed that GBP-1 belongs to the group of large GTP-binding proteins such as Mx and dynamin, all of which have a similar domain composition and GTPase activity, although sequence homology is very low.[20]

We have shown that GBP-1 is the key and selective mediator of the anti-proliferative effect of ICs on both microvascular and macrovascular endothelial cells *in vitro* and that GBP-1 expression was inversely related with cell proliferation in vessel endothelial cells of Kaposi's sarcoma (KS) *in vivo*. In addition, GBP-1 expression in endothelial cells was found to be highly induced by ICs and inhibited by angiogenic growth factors.[22]

The latter findings suggested that GBP-1 may characterize the inflammatory endothelial cell phenotype. In this report we showed that GBP-1 expression in endothelial cells is selectively induced by ICs but by none of several other cytokines, chemokines, and growth factors examined. In addition, we found that GBP-1 was highly associated with endothelial cells in a broad range of human tissues and we demonstrated that GBP-1 characterizes the IC-activated phenotype of endothelial cells in human inflammatory skin diseases.

Materials and Methods

Tissues

All tissues including 37 cases of acquired immune deficiency syndrome (AIDS)-associated KS, two cases of adverse drug reaction of the skin, three cases of psoriasis, and a wide range of normal tissues (Table 1) were routinely processed, formalin-fixed, paraffin-embedded specimens retrieved from the archive files of the Department of Dermatology, University of Vienna Medical School, Vienna, Austria; from the Institute of Pathology, Ludwig Maximilians University, Munich, Germany; and from commercial suppliers (multitissue control slides; Biogenex, San Ramon, CA).

Cell Culture

Human primary fibroblasts and the keratinocyte cell line HaCaT were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate (Gibco BRL). The T-cell line HuT 78, the B-cell line Schlicht, and the monocytic cell line U937 were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate. Both primary human macrovascular umbilical vein endothelial cells (HUVECs) and primary microvascular endothelial cells (MVEC) were purchased from Clonetics (San Diego, CA) and grown in endothelial cell basal medium (Clonetics) supplemented with 5% FBS and propagated in Roux flasks (Greiner, Frickenhausen, Germany) coated with 1.5% bovine skin gelatin, type B (Sigma-Aldrich Chemie, Deisenhofen, Germany) in phosphate-buffered saline (PBS). Fresh peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors by Percoll (Amersham Pharmacia Biotech, Freiburg, Germany) gradient centrifugation and cultivated in RPMI 1640 containing 10% FBS, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate.

For stimulation experiments, cells were maintained in the appropriate medium containing 0.5% FBS for 16 hours, with the exception of peripheral blood mononuclear cells that were kept in full medium. Subsequently, growth factors, cytokines, and chemokines were added in the respective medium containing 0.5% FBS. Cells were harvested after the stimulation period by centrifugation or by trypsinization (0.5 g/L trypsin and 0.2 g/L ethylenediaminetetraacetic acid in Hank's balanced salt solution; Gibco BRL) and subsequent centrifugation. Cellular proteins were extracted with RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium desoxycholate, 1% Nonidet P-40, 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; Roche, Mannheim, Germany).

IL-1α, MCP-1, MIP-1β, PF4, IP-10, SDF-1α, and oncostatin M were purchased from Promocell (Heidelberg, Germany); IFN-γ, bFGF, IL-1β, TNF-α, platelet-derived growth factor (PDGF) B/B, IL-4, IL-6, and IL-10 from Roche; VEGF and angiopoietin-2 (Ang-2) from R&D Systems (Abingdon, UK); and IL-18 from Medical & Biological Laboratories (Nagoya, Japan).

Cloning, Expression, and Purification of Recombinant GBP-1

The coding region of GBP-1 (GenBank accession no. M55542) was amplified by polymerase chain reaction using 4 U of *rTth* DNA polymerase (Perkin Elmer, Wellesley, MA), 8 μmol/L of dNTPs, 7 μmol/L of magnesium acetate, and 0.3 μmol/L of primers, from the full-length GBP-1 cDNA and cloned into the pCR-Script vector (Stratagene, La Jolla, CA). Primers containing restriction sites (bold letters) for cloning had the following sequence: 5′-CG**GGATCC**G**GAATTC**CTGCATCAGAGATCCACATG-3′ (forward primer) and 5′-CT**AGATCTGAGCTC**GCTTATGGTACATGCCTTTCG-3′ (reverse primer). The polymerase chain reaction product was purified using the QIAquick polymerase chain reaction purification kit (Qiagen, Hilden, Germany), digested with *Bam*HI and *Bgl*II (Roche) and cloned into the *Bam*HI site of the pQE-60 expression vector (Qiagen). The correct sequence was confirmed by sequencing.

Recombinant GBP-1-His (6XHis tag at C-terminus), His-GBP-1 (6XHis tag at N-terminus), His-GBP-2, and His-eGFP were produced in *Escherichia coli* M15 strains

(Qiagen) and purified under native conditions using standard NiNTA affinity Sepharose column chromatography. The cells were grown in 500 ml of LB (Luria-Bertani) medium supplemented with ampicillin (100 μg/ml, Sigma) and kanamycin (25 μg/ml, Sigma) at 37°C until an A_{600} value of 0.6 was reached. Subsequently, isopropyl-β-D-thiogalactopyranoside (Qiagen) was added to a final concentration of 100 μmol/L and the culture was grown for an additional 4 hours. The cell pellets were resuspended in 5 ml of 50 mmol/L NaH_2PO_4, 500 mmol/L NaCl, 10% glycerol, 2 mmol/L Tris(2-carboxyethyl)phosphine (TCEP, Sigma), 20 mmol/L imidazole, pH 7.4, and lysed by two passages at 1200 psi through a French press apparatus (SLM Aminco; Polytec, Waldbronn, Germany). The cell lysates were centrifuged at 37,000 × *g* for 60 minutes. The supernatants were filtered (45-μm Ø filters; Millipore, Eschborn, Germany) and subjected to NiNTA Sepharose column chromatography using a fast performance liquid chromatography (FPLC) apparatus (Bio-Rad, Munich, Germany). His-tagged proteins were eluted with a 20- to 250-mmol/L imidazole gradient.

Production of Rat Monoclonal Antibodies

Purified recombinant GBP-1-His protein (50 μg) was injected intraperitoneally and subcutaneously into LOU/C rats. Two months later a final boost was given intraperitoneally and subcutaneously 3 days before sacrifice. Fusion of P3X63-Ag8.653 myeloma cells with the rat spleen cells was performed according to the standard procedure.[23] Hybridoma supernatants were tested in a solid-phase immunoassay using the GBP-1-His fusion protein adsorbed to polystyrene microtiter plates and reactive colonies were stabilized by recloning through a limiting dilution.

Western Blot Analysis

Protein concentrations in cell extracts were determined using the DC protein assay (Bio-Rad). Samples were boiled in Laemmli buffer[24] for 5 minutes, size-separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred to a Hybond-P membrane (Amersham Pharmacia Biotech) at 250 mA for 2 hours. After blocking in 1× Western blocking reagent solution (Roche) and 0.1% Tween 20 (Sigma) in PBS overnight at 4°C, the blots were incubated with rabbit anti-actin antiserum (1:1000, Sigma) and with monoclonal antibody (mAb) 1B1 (1:500). After incubation for 1 hour at room temperature with the primary antibodies the blots were washed and incubated for 45 minutes with goat anti-rat and goat anti-rabbit IgG coupled to horseradish peroxidase (1:5000; Dianova, Hamburg, Germany). All antibodies were diluted in 0.5× Western blocking reagent solution (Roche) containing 0.1% Tween 20 (Sigma). Detection was performed with the enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech).

Immunohistochemistry

Cells were plated in 8-chamber glass culture slides (Becton Dickinson Labware, San Diego, CA) and stimulated as described. After stimulation, the cells were fixed in ethanol (20 minutes, 4°C) washed in PBS and incubated with mAb 1B1 (1:100 in antibody diluent with background reducing component; DAKO, Glostrup, Denmark) for 1 hour. Subsequently, the cells were incubated for 30 minutes with biotinylated horse anti-rat IgG (ABC kit; Vector Laboratories, Burlingame, CA), followed by 30 minutes of incubation with the avidin-biotin complex (ABC kit, Vector Laboratories). The reaction was developed with 3,3′-diaminobenzidine (Biogenex) for 8 minutes at room temperature. Nuclei were counterstained with Gill-3 hematoxylin (Shandon, Pittsburgh, PA) and mounted with Immunomount (Shandon).

Paraffin-embedded sections (6 μm) were dewaxed in xylene and rehydrated. After antigen retrieval by microwave pretreatment (3 times at 7 minutes, 580 W) in target retrieval solution, pH 9 (TRS9; DAKO), the slides were treated with 7.5% hydrogen peroxide for 10 minutes at room temperature to block endogenous peroxidases. Then the slides were incubated for 1 hour with mAb 6F12 or mAb 1B1 (1:300) or with the respective isotype control antibodies (R&D Systems) at room temperature. For controls 1 μl of mAb 1B1 was incubated with 80 μg of purified recombinant GBP-1-His for 2 hours at room temperature. Antibody detection, counterstaining of the nuclei, and mounting was performed as described above. Stained sections were photographed using a charge-coupled device camera (Sony, Cologne, Germany) mounted on an Aristoplan microscope (Leitz, Wetzlar, Germany).

Immunofluorescence

HUVECs were plated in 8-chamber glass culture slides (Becton Dickinson Labware), stimulated as described and fixed in ethanol (20 minutes, 4°C). Slides were incubated with mAb 1B1 (1:100) overnight at 4°C, washed, and incubated with goat anti-rat antibody (1:500) coupled to the fluorochrome AlexaFluor546 (Molecular Probes Europe, Leiden, The Netherlands) for 1 hour at room temperature. Nuclei were visualized with 4′,6′-diamino-2-phenylindole (Molecular Probes Europe) at a final concentration of 1 μg/ml and slides were mounted with 50% glycerol/PBS.

Double-immunofluorescence labeling of tissues was performed as described.[25] Six-μm paraffin-embedded sections were dewaxed in xylene and rehydrated. After antigen retrieval as described above, the slides were blocked with 10% normal goat serum for 10 minutes at room temperature and subsequently incubated overnight at 4°C with a mixture of mAb 1B1 (1:100) and mouse anti-CD31 (1:20; Serotec, Oxford, UK). Afterward a mixture of highly cross-absorbed goat anti-rat and goat anti-mouse antibodies (1:500) coupled to the fluorochromes AlexaFluor 488 (green fluorescence) and AlexaFluor 546 (red fluorescence), respectively, was applied for 1 hour

at room temperature. Confocal microscopy was performed on a scanning microscope (Zeiss, Jena, Germany). Pictures were split into RGB channels using the Corel Photo-Paint 8 software (Corel Corporation, Unterschleissheim, Germany) or the LSM 5 Image Browser (Zeiss).

Results

Induction of GBP-1 Expression by IFN-γ in Different Cell Types In Vitro

Monoclonal anti-GBP-1 antibodies (mAbs) were generated by immunization of LOU/C rats with purified GBP-1-His. Two hybridoma clones were established. Clone 1B1 (IgG_1) secreted a mAb that specifically reacted with GBP-1 (Figure 1A, left). Another mAb was secreted by clone 6F12 (IgG_{2a}) and recognized both GBP-1 and GBP-2 (Figure 1A, right). None of the mAbs reacted with eGFP that was used as a negative control (Figure 1A).

Western blot analysis with mAb 1B1 showed that IFN-γ increases GBP-1 expression in many different cell lines and primary cells including B-cells, monocytes, peripheral blood mononuclear cells, keratinocytes, fibroblasts, and endothelial cells, with the exception of T cells (Figure 1B).

Selective Induction of GBP-1 Expression in Endothelial Cells by Inflammatory Cytokines

To investigate the effect of external stimulations on GBP-1 expression in endothelial cells in a greater detail, HUVECs were treated with cytokines (IFN-γ, IL-1α, IL-1β, TNF-α, IL-4, IL-6, IL-10, IL-18, oncostatin M), chemokines (MCP-1, MIP-1β, PF4, IP-10, SDF-1α), and growth factors (bFGF, VEGF, Ang-2, PDGF B/B).

In the concentration used each factor induced a clear biological response in HUVECs. Endothelial cell proliferation was activated by bFGF, VEGF, and PDGF B/B and inhibited by IFN-γ, IL-1β, TNF-α, oncostatin M, and PF4. IL-4 up-regulated VCAM-1 expression. SDF-1α, MCP-1, and IL-18 induced chemotaxis of the cells and IL-1α down-regulated von Willebrand factor expression (data not shown). All findings were in agreement with previously published activities of these factors on HUVECs.[9–14,22,26–36]

Western blot analysis revealed that under these different conditions GBP-1 expression was selectively induced by ICs including IFN-γ, IL-1α, IL-1β, and TNF-α, but by none of the other factors (Figure 1C).

Induction of GBP-1 expression by ICs was confirmed at the single cell level by immunocytochemistry (Figure 2, A and B) and immunofluorescence (Figure 2, C and D). Both methods produced a strong, exclusively cytoplasmic staining of GBP-1 in IFN-γ-treated (Figure 2, B and D), IL-1β-treated, and TNF-α-treated (data not shown) cells, but not in untreated cells (Figure 2, A and C).

Expression of GBP-1 by Endothelial Cells of Human Tissues

GBP-1 expression was highly associated with endothelial cells in different human tissues (Table 1). Examples of

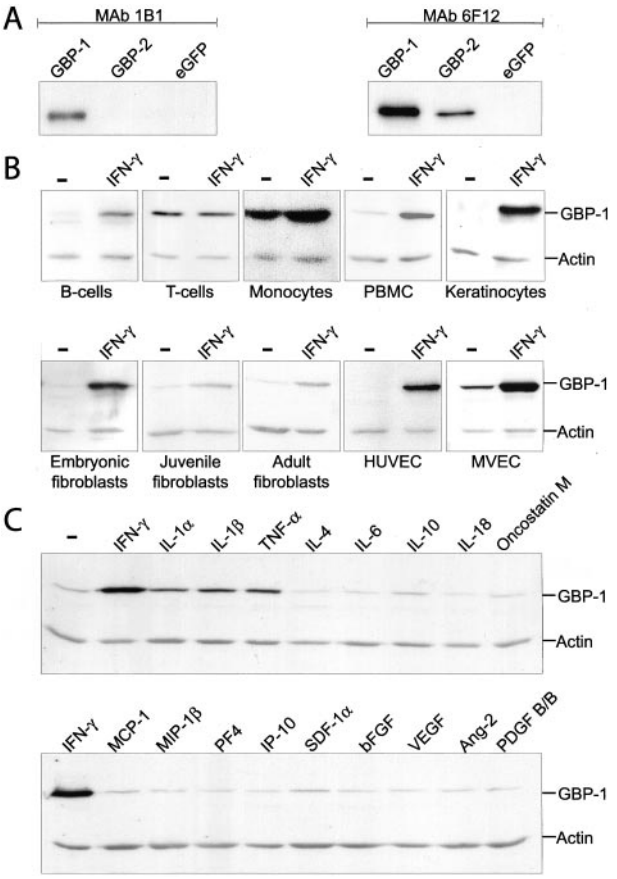

Figure 1. Selective induction of GBP-1 expression in endothelial cells by ICs. **A:** Purified recombinant GBP-1, GBP-2, and eGFP (100 ng each) were detected by Western blot using mAb 1B1 and mAb 6F12. **B:** Western blot analysis of GBP-1 expression in different cell types that were either untreated (−) or stimulated with IFN-γ (100 U/ml) for 16 hours. **C:** Western blot analysis of GBP-1 expression in HUVECs stimulated with the indicated factors for 24 hours. 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; oncostatin M, 10 ng/ml; MCP-1, 50 ng/ml; PF4, 25 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. It was confirmed by our experiments and it is according to the literature that in the concentrations used each factor induces a significant cell biological response in these cells.[9–14,22,26–36,56,57,64,65,71] In addition IP-10 (50 ng/ml) was used in a concentration similar to that established in the supernatant of IFN-γ-stimulated endothelial cells.[72] MIP-1β (50 ng/ml) in the concentration used exerts maximal chemotactic activity on human T cells.[73] mAb 1B1 was used in **B** and **C**. Immunochemical detection of actin (Actin) demonstrated that equal amounts of proteins were blotted onto the membranes.

immunohistochemical detection of GBP-1 expression in endothelial cells of spleen, uterus, lung, and heart are shown in Figure 3; A to D, black arrows. GBP-1 was not detected in endothelial cells of the skin (Figure 3, E and F, negative vessels are indicated by a white arrow).

An antibody that detected GBP-1 and GBP-2 (mAb 6F12) (Figure 3I) produced similar staining patterns in the various tissues as compared to mAb 1B1 [compare Figure 3A (mAb 1B1) and Figure 3I (mAb 6F12)]. This indicated that GBP-1 is the major isoform expressed in endothelial cells.

In addition to endothelial cells, GBP-1 expression was detected in mononuclear cells in the bladder, lung, stomach, colon, and liver and in the epithelium in prostate, lung, colon, stomach, and thyroid as assessed by morphological analysis (Table 1).

Table 1. Expression of GBP-1 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.

Control stainings with a primary antibody that had been preadsorbed with GBP-1-His protein did not produce any signals (Figure 3G, lung). In addition, no signals were obtained when the staining procedure was performed without the primary antibody (data not shown) or with an isotype control antibody (Figure 3H, spleen).

Simultaneous detection of GBP-1 (Figure 4, left) and the endothelial cell-associated antigen CD31 (Figure 4, middle) in tissue sections of bladder (Figure 4A), endometrium (Figure 4B), heart (Figure 4C), and lung (Figure 4D) confirmed that GBP-1 was highly associated with endothelial cells in human tissues (Figure 4; GBP-1: green, left; CD31: red, middle; co-localization: yellow, right, white arrows).

GBP-1 Expression in Endothelial Cells in Diseases of the Skin with a High-Inflammatory Component

To determine whether GBP-1 expression is induced by ICs in endothelial cells *in vivo*, GBP-1 expression was

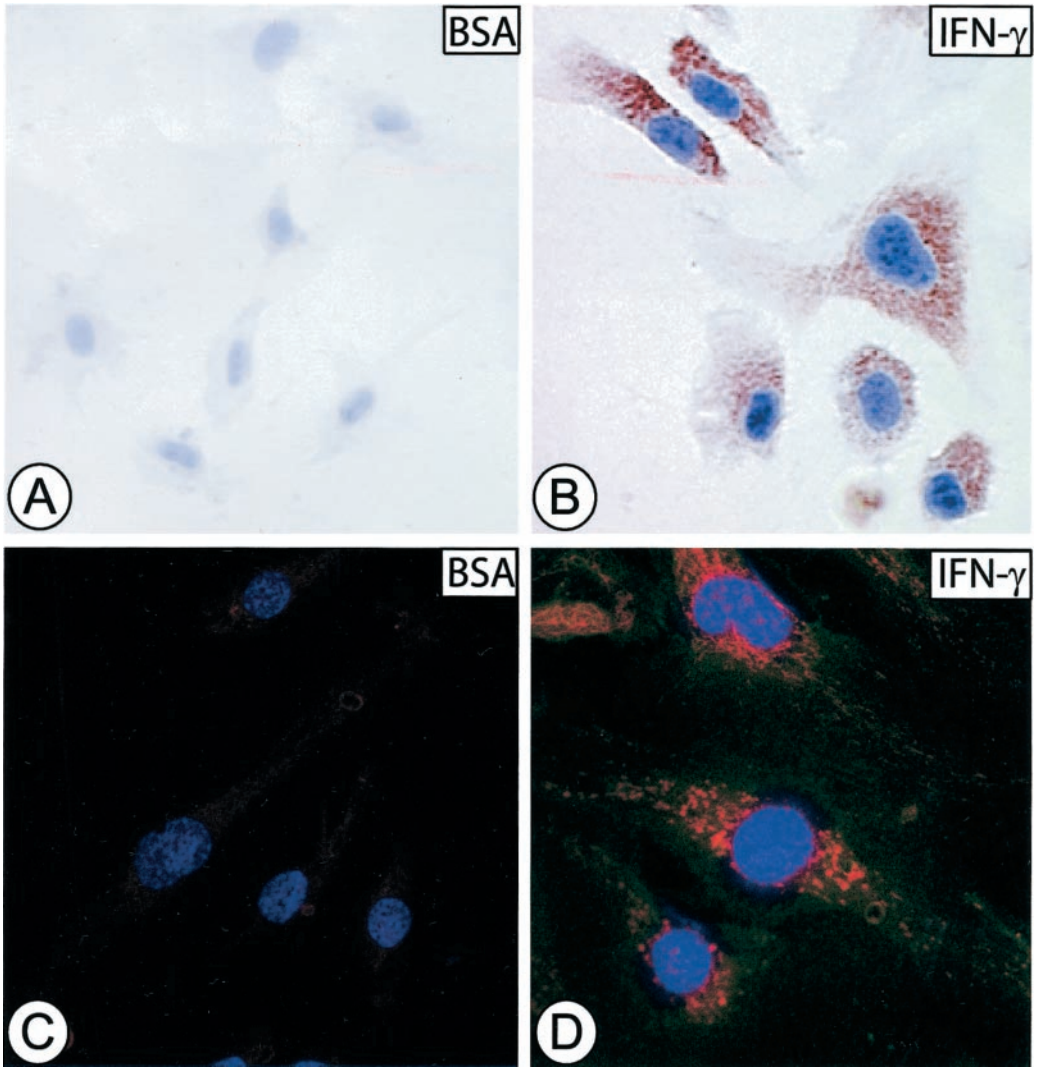

Figure 2. Localization of GBP-1 in the cytoplasm of endothelial cells. GBP-1 expression in HUVECs was analyzed by standard ABC immunoperoxidase staining (**A**, **B**) or by indirect immunofluorescence (**C**, **D**) using mAb 1B1 before (**A**, **C**) or after (**B**, **D**) stimulation with 100 U/ml IFN-γ for 16 hours. Original magnifications: ×450 (**A**, **B**); ×630 (**C**, **D**).

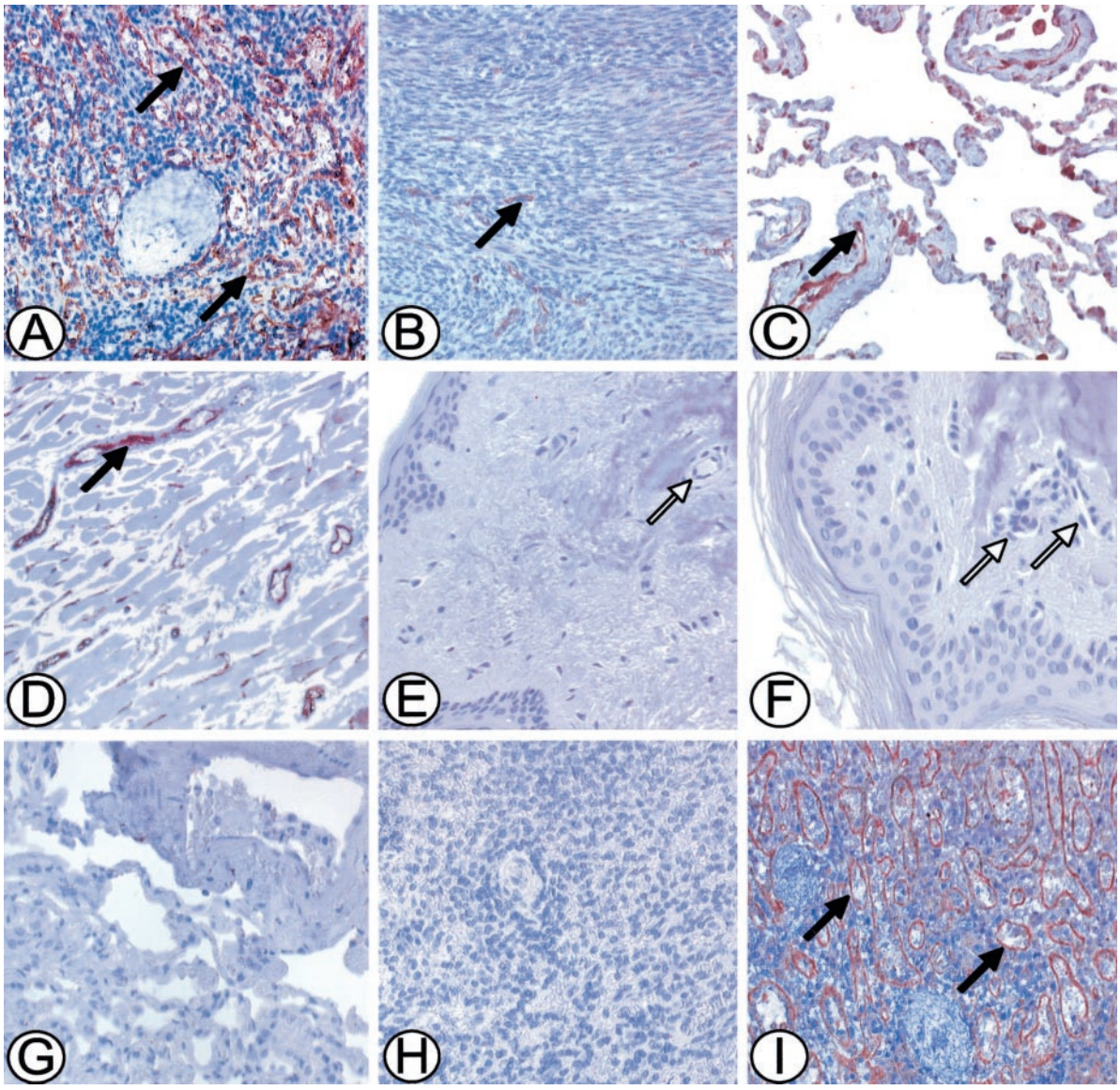

Figure 3. Expression of GBP-1 in blood vessels of various human tissues. Detection of GBP-1 expression using the standard ABC immunohistochemical method in paraffin sections of spleen (**A**, **I**), uterus (**B**), lung (**C**), heart (**D**), and skin (**E**, **G**). Controls: preadsorption of mAb 1B1 with an excess (300-fold) of purified GBP-1-His (**G**, lung) and staining with an isotypic control antibody (**H**, spleen); mAb 1B1 was used in **A–F** and mAb 6F12 was used in **I**. Examples of GBP-1-positive (**black arrows**) and -negative (**white arrows**) vessels are indicated. Original magnifications, ×250.

investigated in AIDS-associated KS (n = 37, Figure 5, B and E), adverse drug reactions of the skin (n = 2, Figure 5C), and psoriasis (n = 3, Figure 5D) that express high levels of ICs.[22,37–44]

Notably, GBP-1 was detected in the inflammatory skin diseases and in KS (Figure 5; B to E, left) but not in healthy skin (Figure 5A, left, compare also Figure 3, E and F). In addition, in diseased skin, GBP-1 was selectively expressed in endothelial cells as indicated by simultaneous detection of GBP-1 and CD31 (Figure 5; A to D; GBP-1: green, left; CD31: red, middle; co-localization: yellow, right, white arrows). The specificity of the cross-absorbed secondary antibodies is demonstrated by the facts that GBP-1-negative skin sections did not show any green fluorescence of the anti-rat antibody-coupled fluorochrome (Figure 5A, left) and that KS sections that were stained without the anti-CD31 antibody did not reveal any red fluorescence of the anti-murine antibody-coupled fluorochrome (Figure 5E, middle).

GBP-1 expression was detected in small size vessels in adverse drug reactions of the skin (Figure 5C), in intermediate size vessels in KS (Figure 5, B and E), and in larger vessels in psoriasis (Figure 5D). In all specimens GBP-1 expression was restricted to single vessels (Figure 5; B to E). This is in agreement with the locally restricted expression of ICs that has been reported in all of these lesions.[22,37–44]

Discussion

The gene encoding human GBP-1 is among the major IFN-γ-induced genes.[15,16,45–48] In fact, GBP-1 mRNA ex-

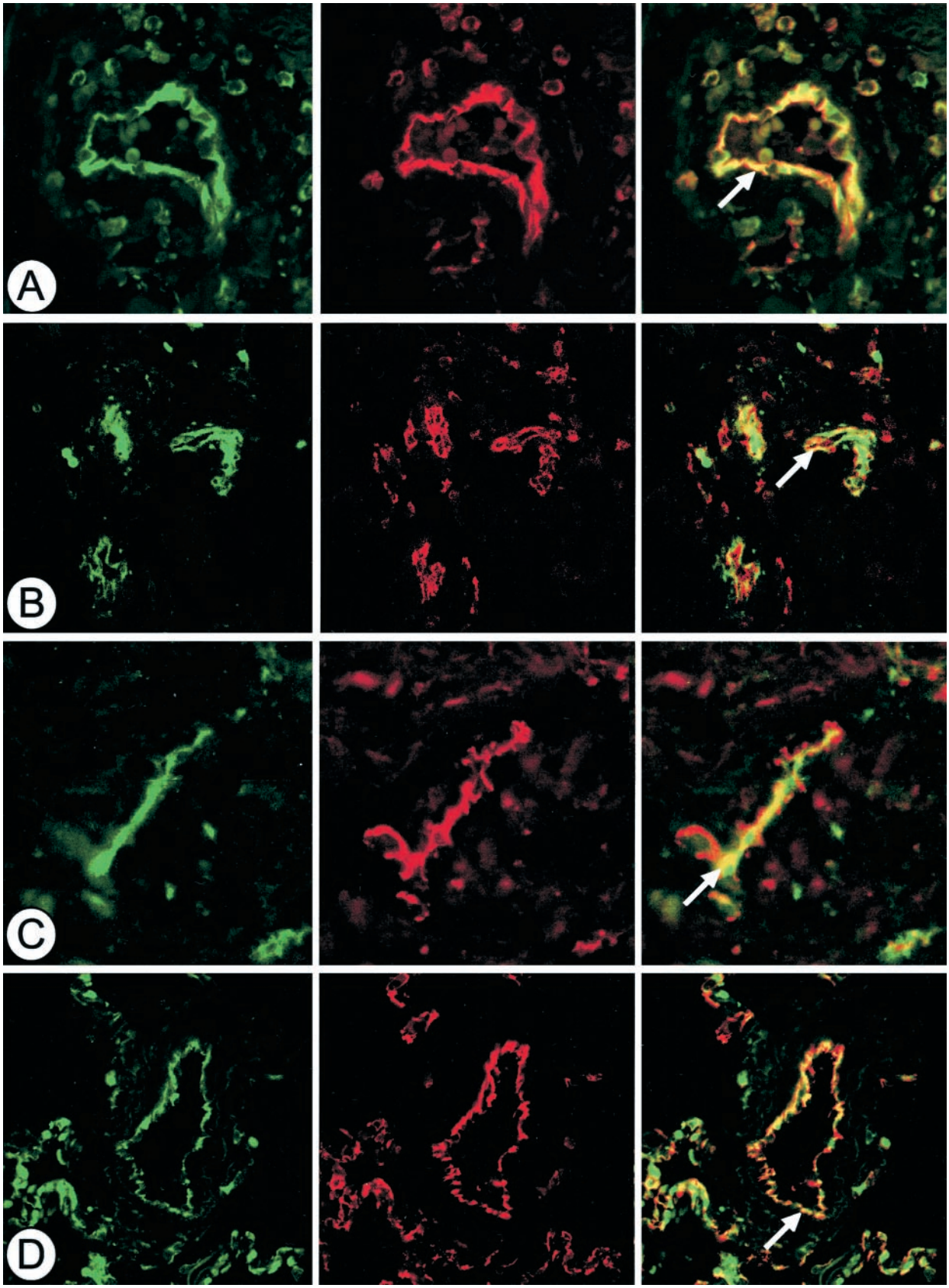

Figure 4. Expression of GBP-1 in vascular endothelial cells in human tissues. Indirect immunofluorescence staining of tissue sections of bladder (**A**), endometrium (**B**), heart (**C**), and lung (**D**) for GBP-1 (green, **left**) and the endothelial cell-associated antigen CD31 (red, **middle**). Merging of the two pictures (**right**) shows co-localization of GBP-1 and CD31 (yellow, **white arrows**). Original magnifications, ×400.

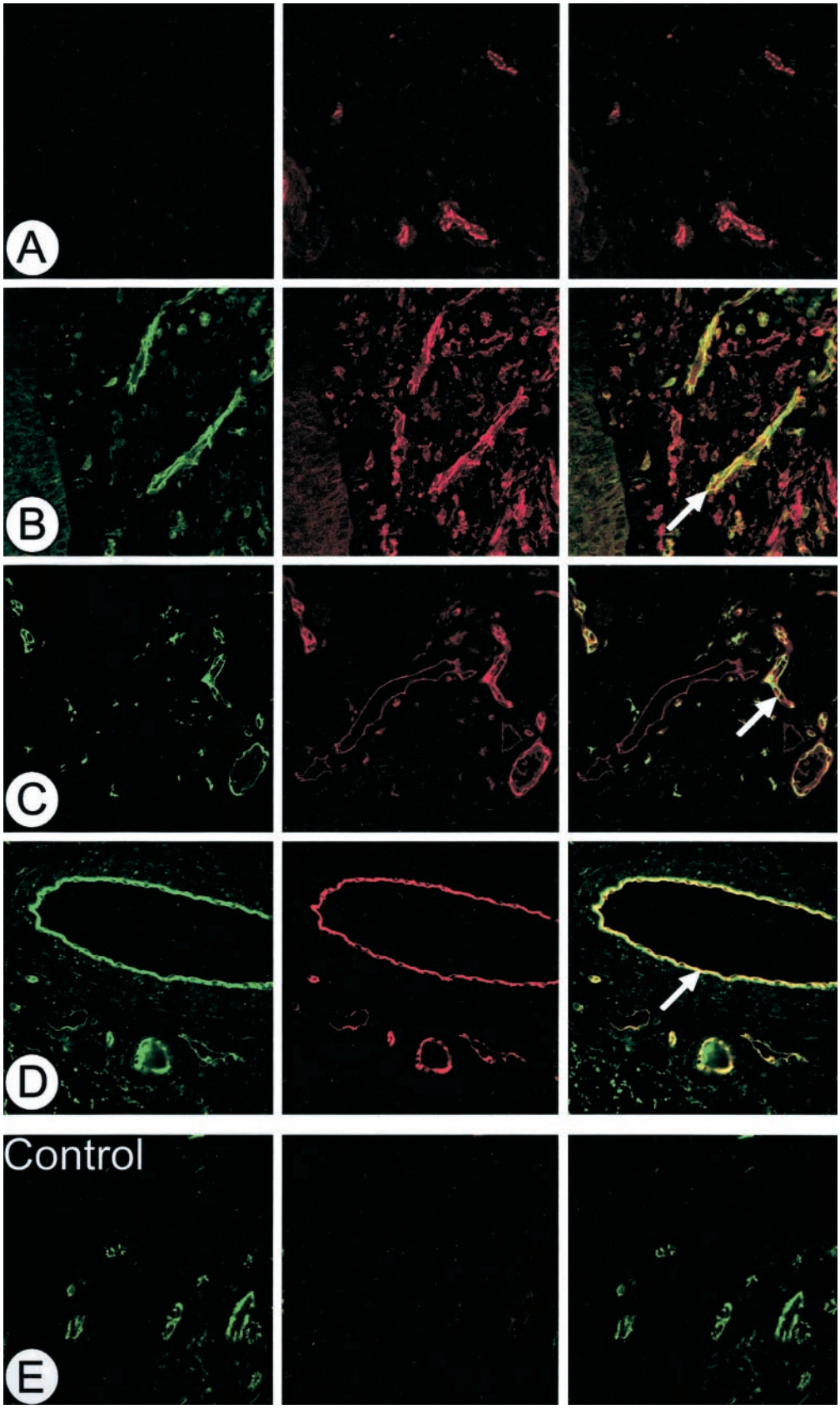

Figure 5. Induction of GBP-1 expression in vascular endothelial cells in diseases of the skin with a high-inflammatory component. Indirect immunofluorescence staining of tissue sections of healthy skin (**A**), KS (**B**, **E**), adverse drug reaction of the skin (**C**), and psoriasis (**D**) for GBP-1 (green, **left**, **A–E**) and the endothelial cell-associated antigen CD31 (red, **middle**, **A–D**). Merging of the two pictures (**right**) shows co-localization (yellow, **white arrows**). Original magnifications: ×250 (**A**, **B**, **E**); ×400 (**C**, **D**).

pression has often been used as a marker to demonstrate IFN-γ activation of cells in culture.[49–53] However, GBP-1 protein expression has not been investigated in detail as yet. Here we identified GBP-1 as a novel molecular marker of the IC-activated phenotype of endothelial cells *in vitro* and *in vivo*. This was accomplished by the development of an antibody for the detection of GBP-1 by Western blot analysis and in paraffin sections.

By use of this antibody we confirmed at the protein level that GBP-1 expression *in vitro* can be induced by IFN-γ in a variety of eukaryotic cells including B cells, monocytes, keratinocytes, fibroblasts, and endothelial cells.[15,46–48]

Endothelial cells express receptors for many different cytokines,[54–60] chemokines,[26,34,61–63] and growth factors[7,64,65] that mediate phenotypic changes of these cells to the respective factors. Consequently, we tested several cytokines, chemokines, and growth factors for their ability to increase GBP-1 expression in HUVECs. We found that in addition to IFN-γ, selectively IL-1α, IL-1β, and TNF-α increased GBP-1 expression in endothelial cells but none of the other factors. Notably, all of the ICs, which induced GBP-1 expression in endothelial cells, have been shown to inhibit proliferation of these cells[22,66–69] and to induce in these cells adhesiveness for monocytes.[13,14] Therefore, GBP-1 expression characterizes an IC-induced nonproliferative, adhesion-competent phenotype of endothelial cells.[22]

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 endothelial cells *in vivo*. This was confirmed by double-labeling studies for simultaneous detection of the endothelial cell-associated antigen CD31 and GBP-1.

Endothelial cells in healthy skin did not express GBP-1. We took advantage of this fact to determine whether GBP-1 expression in endothelial cells *in vivo* may be induced by ICs. To this goal GBP-1 expression was investigated in three different vascularized and IC-regulated diseases of the skin, namely KS, psoriasis, and adverse drug reactions of the skin.

KS is an angioproliferative disease whose most aggressive form, AIDS-associated KS, is associated with infection of two different viruses (human herpesvirus-8 and human immunodeficiency virus-1).[44,70] The tumorigenic interplay of these two viruses is mediated by the same ICs that have been shown to induce GBP-1 expression, and expression of all these cytokines has been demonstrated in KS tissue sections.[22,37–44] In addition, in psoriasis and adverse drug reactions of the skin IFN-γ expression has been detected.[39–42]

In contrast to healthy skin, GBP-1 was detected in each of the three different diseases of the skin with a high-inflammatory component. GBP-1 was selectively expressed in vascular endothelial cells and positive cells were restricted to single vessels (Figure 5; B to D). The latter finding is well in agreement with the locally restricted expression of ICs in these diseases.[22,37–44]

In summary, we show that GBP-1 expression is highly associated with endothelial cells *in vivo* and is selectively up-regulated by ICs in inflammatory skin diseases. These findings indicate that GBP-1 is a novel activation marker that characterizes the IC-activated phenotype of endothelial cells. The anti-GBP-1 antibody described here will be a useful tool to determine the temporal and spatial appearance of the IC-activated phenotype of endothelial cells in physiological and pathophysiological angiogenesis. This will help to decipher the multicellular and multifactorial interactions regulating angiogenesis and may provide a platform for the development of novel anti- and pro-angiogenic approaches targeting distinct activation phenotypes of endothelial cells.

Acknowledgments

We thank Judith Johnson (Institute of Immunology, Ludwig Maximilians University Munich, Germany) for providing the B-cell line and Michael H. Lehmann (Department of Virus-Induced Vasculopathy, GSF-National Research Center for Environment and Health, Neuherberg, Germany) for critically reading the manuscript.

References

1. 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, Stern DM: Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998, 91:3527–3561
2. Pober JS: Warner-Lambert/Parke-Davis award lecture. Cytokine-mediated activation of vascular endothelium. Physiology and pathology. Am J Pathol 1988, 133:426–433
3. Cotran RS, Pober JS: Endothelial Activation: Its Role in Inflammatory and Immune Reactions. New York, Plenum Press, 1988, pp 335–347
4. Roesen P, Ferber P, Tschoepe D: Macrovascular disease in diabetes: current status. Exp Clin Endocrinol Diabetes 2001, 109(Suppl):S474–S486
5. Baumgartl HJ, Standl E: The atherosclerotic process and its exacerbation by diabetes. Exp Clin Endocrinol Diabetes 2001, 109(Suppl): S487–S492
6. Cotran RS, Pober JS: Cytokine-endothelial interactions in inflammation, immunity, and vascular injury. J Am Soc Nephrol 1990, 1:225–235
7. Plate KH, Breier G, Weich HA, Risau W: Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 1992, 359:845–848
8. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N: Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 1993, 362:841–844
9. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT: Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989, 246:1309–1312
10. Ferrara N, Henzel WJ: Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989, 161:851–858
11. Folkman J, Klagsbrun M: Angiogenic factors. Science 1987, 235: 442–447
12. Cornali E, Zietz C, Benelli R, Weninger W, Masiello L, Breier G, Tschachler E, Albini A, Stürzl M: Vascular endothelial growth factor regulates angiogenesis and vascular permeability in Kaposi's sarcoma. Am J Pathol 1996, 149:1851–1869
13. Bevilacqua MP, Stengelin S, Gimbrone Jr MA, Seed B: Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 1989, 243:1160–1165
14. Cavender DE, Edelbaum D, Welkovich L: 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 1991, 49:566–578
15. Cheng YS, Colonno RJ, Yin FH: Interferon induction of fibroblast proteins with guanylate binding activity. J Biol Chem 1983, 258:7746–7750
16. Cheng YS, Patterson CE, Staeheli P: Interferon-induced guanylate-binding proteins lack an N(T)KXD consensus motif and bind GMP in addition to GDP and GTP. Mol Cell Biol 1991, 11:4717–4725
17. Praefcke GJ, Geyer M, Schwemmle M, Robert Kalbitzer H, Herrmann C: Nucleotide-binding characteristics of human guanylate-binding protein 1 (hGBP1) and identification of the third GTP-binding motif. J Mol Biol 1999, 292:321–332
18. Neun R, Richter MF, Staeheli P, Schwemmle M: GTPase properties of the interferon-induced human guanylate-binding protein 2. FEBS Lett 1996, 390:69–72
19. Schwemmle M, Staeheli P: The interferon-induced 67-kDa guanylate-binding protein (hGBP1) is a GTPase that converts GTP to GMP. J Biol Chem 1994, 269:11299–11305
20. Prakash B, Praefcke GJ, Renault L, Wittinghofer A, Herrmann C: Structure of human guanylate-binding protein 1 representing a unique class of GTP-binding proteins. Nature 2000, 403:567–571
21. Prakash B, Renault L, Praefcke GJ, Herrmann C, Wittinghofer A: Triphosphate structure of guanylate-binding protein 1 and implications for nucleotide binding and GTPase mechanism. EMBO J 2000, 19:4555–4564
22. 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, Stürzl M: The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. EMBO J 2001, 20:5568–5577
23. Galfre G, Howe SC, Milstein C, Butcher GW, Howard JC: Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 1977, 266:550–552
24. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227:680–685
25. Mason DY, Micklem K, Jones M: Double immunofluorescence labelling of routinely processed paraffin sections. J Pathol 2000, 191:452–461
26. Gentilini G, Kirschbaum NE, Augustine JA, Aster RH, Visentin GP: 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 1999, 93:25–33
27. Takashima S, Klagsbrun M: 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 1996, 271:24901–24906
28. Battegay EJ, Rupp J, Iruela-Arispe L, Sage EH, Pech M: PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. J Cell Biol 1994, 125:917–928
29. Lee YW, Kuhn H, Hennig B, Neish AS, Toborek M: IL-4-induced oxidative stress upregulates VCAM-1 gene expression in human endothelial cells. J Mol Cell Cardiol 2001, 33:83–94
30. Swerlick RA, Lee KH, Li LJ, Sepp NT, Caughman SW, Lawley TJ: Regulation of vascular cell adhesion molecule 1 on human dermal microvascular endothelial cells. J Immunol 1992, 149:698–705
31. Haraldsen G, Kvale D, Lien B, Farstad IN, Brandtzaeg P: 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 1996, 156:2558–2565
32. Holzinger C, Weissinger E, Zuckermann A, Imhof M, Kink F, Schollhammer A, Kopp C, Wolner E: Effects of interleukin-1, -2, -4, -6, interferon-gamma and granulocyte/macrophage colony stimulating factor on human vascular endothelial cells. Immunol Lett 1993, 35:109–117
33. Romero LI, Zhang DN, Herron GS, Karasek MA: Interleukin-1 induces major phenotypic changes in human skin microvascular endothelial cells. J Cell Physiol 1997, 173:84–92
34. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ: Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood 2000, 96:34–40
35. Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, Kleinman HK, Murphy WJ, Oppenheim JJ: 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 1999, 154:1125–1135
36. Park CC, Morel JC, Amin MA, Connors MA, Harlow LA, Koch AE: Evidence of IL-18 as a novel angiogenic mediator. J Immunol 2001, 167:1644–1653
37. Stürzl M, Brandstetter H, Zietz C, Eisenburg B, Raivich G, Gearing DP, Brockmeyer NH, Hofschneider PH: 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 1995, 10:2007–2016
38. Fiorelli V, Gendelman R, Sirianni MC, Chang HK, Colombini S, Markham PD, Monini P, Sonnabend J, Pintus A, Gallo RC, Ensoli B: 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 1998, 91:956–967
39. Ackermann L, Harvima IT, Pelkonen J, Ritamaki-Salo V, Naukkarinen A, Harvima RJ, Horsmanheimo M: Mast cells in psoriatic skin are strongly positive for interferon-gamma. Br J Dermatol 1999, 140:624–633
40. Gottlieb AB, Luster AD, Posnett DN, Carter DM: Detection of a gamma interferon-induced protein IP-10 in psoriatic plaques. J Exp Med 1988, 168:941–948
41. Hari Y, Urwyler A, Hurni M, Yawalkar N, Dahinden C, Wendland T, Braathen LR, Matter L, Pichler WJ: Distinct serum cytokine levels in drug- and measles-induced exanthema. Int Arch Allergy Immunol 1999, 120:225–229
42. Kapp A: The role of cytokines in the psoriatic inflammation. J Dermatol Sci 1993, 5:133–142
43. Ensoli B, Sgadari C, Barillari G, Sirianni MC, Stürzl M, Monini P: Biology of Kaposi's sarcoma. Eur J Cancer 2000, 37:1251–1269
44. Stürzl M, Zietz C, Monini P, Ensoli B: Human herpesvirus-8 and Kaposi's sarcoma: relationship with the multistep concept of tumorigenesis. Adv Cancer Res 2001, 81:125–159
45. Cheng YS, Becker-Manley MF, Chow TP, Horan DC: Affinity purification of an interferon-induced human guanylate-binding protein and its characterization. J Biol Chem 1985, 260:15834–15839
46. Decker T, Lew DJ, Cheng YS, Levy DE, Darnell Jr JE: Interactions of alpha- and gamma-interferon in the transcriptional regulation of the gene encoding a guanylate-binding protein. EMBO J 1989, 8:2009–2014
47. Nantais DE, Schwemmle M, Stickney JT, Vestal DJ, Buss JE: Prenylation of an interferon-gamma-induced GTP-binding protein: the human guanylate binding protein, huGBP1. J Leukoc Biol 1996, 60:423–431
48. Saunders NA, Smith RJ, Jetten AM: Regulation of guanylate-binding protein expression in interferon-gamma-treated human epidermal keratinocytes and squamous cell carcinoma cells. J Invest Dermatol 1999, 112:977–983
49. Kumar S, Li Q, Dua A, Ying YK, Bagchi MK, Bagchi IC: 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 2001, 86:2420–2427
50. Tnani M, Bayard BA: Evidence for IRF-1-dependent gene expression deficiency in interferon unresponsive HepG2 cells. Biochim Biophys Acta 1999, 1451:59–72
51. van Loon AP, Ozmen L, Fountoulakis M, Kania M, Haiker M, Garotta G: 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 1991, 49:462–473
52. Yang E, Wen Z, Haspel RL, Zhang JJ, Darnell Jr JE: The linker domain of Stat1 is required for gamma interferon-driven transcription. Mol Cell Biol 1999, 19:5106–5112
53. Ucer U, Bartsch H, Scheurich P, Berkovic D, Ertel C, Pfizenmaier K: Quantitation and characterization of gamma-interferon receptors on human tumor cells. Cancer Res 1986, 46:5339–5343
54. Madge LA, Pober JS: TNF signaling in vascular endothelial cells. Exp Mol Pathol 2001, 70:317–325
55. Thornhill MH, Haskard DO: IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-gamma. J Immunol 1990, 145:865–872
56. Thornhill MH, Kyan-Aung U, Haskard DO: IL-4 increases human

endothelial cell adhesiveness for T cells but not for neutrophils. J Immunol 1990, 144:3060–3065
57. Maruo N, Morita I, Shirao M, Murota S: IL-6 increases endothelial permeability in vitro. Endocrinology 1992, 131:710–714
58. Mallat Z, Corbaz A, Scoazec A, Besnard S, Leseche G, Chvatchko Y, Tedgui A: Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. Circulation 2001, 104: 1598–1603
59. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A: Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001, 19:683–765
60. Vasse M, Pourtau J, Trochon V, Muraine M, Vannier JP, Lu H, Soria J, Soria C: Oncostatin M induces angiogenesis in vitro and in vivo. Arterioscler Thromb Vasc Biol 1999, 19:1835–1842
61. Sanders VJ, Pittman CA, White MG, Wang G, Wiley CA, Achim CL: Chemokines and receptors in HIV encephalitis. Aids 1998, 12:1021–1026
62. Murdoch C, Monk PN, Finn A: Cxc chemokine receptor expression on human endothelial cells. Cytokine 1999, 11:704–712
63. Dzenko KA, Andjelkovic AV, Kuziel WA, Pachter JS: The chemokine receptor CCR2 mediates the binding and internalization of monocyte chemoattractant protein-1 along brain microvessels. J Neurosci 2001, 21:9214–9223
64. Thommen R, Humar R, Misevic G, Pepper MS, Hahn AW, John M, Battegay EJ: PDGF-BB increases endothelial migration on cord movements during angiogenesis in vitro. J Cell Biochem 1997, 64: 403–413
65. 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, Yancopoulos GD: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997, 277:55–60
66. Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P: Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. Proc Natl Acad Sci USA 1987, 84:5277–5281
67. Friesel R, Komoriya A, Maciag T: Inhibition of endothelial cell proliferation by gamma-interferon. J Cell Biol 1987, 104:689–696
68. Schweigerer L, Malerstein B, Gospodarowicz D: Tumor necrosis factor inhibits the proliferation of cultured capillary endothelial cells. Biochem Biophys Res Commun 1987, 143:997–1004
69. Cozzolino F, Torcia M, Aldinucci D, Ziche M, Almerigogna F, Bani D, Stern DM: Interleukin 1 is an autocrine regulator of human endothelial cell growth. Proc Natl Acad Sci USA 1990, 87:6487–6491
70. Stürzl M, Hohenadl C, Zietz C, Castanos-Velez E, Wunderlich A, Ascherl G, Biberfeld P, Monini P, Browning PJ, Ensoli B: Expression of K13/v-FLIP gene of human herpesvirus 8 and apoptosis in Kaposi's sarcoma spindle cells. J Natl Cancer Inst 1999, 91:1725–1733
71. Audero E, Cascone I, Zanon I, Previtali SC, Piva R, Schiffer D, Bussolino F: Expression of angiopoietin-1 in human glioblastomas regulates tumor-induced angiogenesis: in vivo and in vitro studies. Arterioscler Thromb Vasc Biol 2001, 21:536–541
72. Piali L, Weber C, LaRosa G, Mackay CR, Springer TA, Clark-Lewis I, Moser B: The chemokine receptor CXCR3 mediates rapid and shear-resistant adhesion-induction of effector T lymphocytes by the chemokines IP10 and Mig. Eur J Immunol 1998, 28:961–972
73. Mashikian MV, Ryan TC, Seman A, Brazer W, Center DM, Cruikshank WW: Reciprocal desensitization of CCR5 and CD4 is mediated by IL-16 and macrophage-inflammatory protein-1 beta, respectively. J Immunol 1999, 163:3123–3130