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Angiostatic immune reaction in colorectal carcinoma: Impact on survival and perspectives for antiangiogenic therapy

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Angiogenesis and inflammation are the 2 major stroma reactions in colorectal carcinoma (CRC). Guanylate binding protein-1 (GBP-1) is a key mediator of angiostatic effects of inflammation. Therefore, we hypothesized that GBP-1 may be a biomarker of intrinsic angiostasis associated with an improved outcome in CRC patients. GBP-1 was strongly expressed in endothelial cells and immune cells in the desmoplastic stroma of 32% of CRC as determined by immunohistochemical investigation of 388 sporadic CRC. Cancer-related 5-year survival was highly significant ($p < 0.001$) increased (16.2%) in patients with GBP-1-positive CRC. Multivariate analysis showed that GBP-1 is an independent prognostic factor indicating a reduction of the relative risk of cancer-related death by the half ($p = 0.032$). A comparative transcriptome analysis (22,215 probe sets) of GBP-1-positive ($n = 12$) and -negative ($n = 12$) tumors showed that particularly IFN-γ-induced genes including the major antiangiogenic chemokines CXCL9, CXCL10 and CXCL11 were coexpressed with GBP-1. Altogether our findings indicated that GBP-1 may be a novel biomarker and an active component of a Th-1-like angiostatic immune reaction in CRC. This reaction may affect patient's response to antiangiogenic therapy and the identification of such tumors may provide a novel criterion for patient selection. Moreover, the induction of a Th-1-like angiostatic immune reaction may be a promising approach for the clinical treatment of CRC.

Key words: guanylate binding protein-1; interferon-γ; colorectal carcinoma; angiogenesis; inflammation

Colorectal carcinoma (CRC) is the second most common malignant tumor, both in men and women, in the western world.[1] Angiogenesis and inflammation are the 2 primary stroma reactions involved in CRC pathogenesis.[2–4]

The high impact of angiogenesis on the growth of cancer has been convincingly demonstrated in numerous animal studies.[5,6] Recently, the inhibition of blood vessel growth has also been successfully applied in human cancer therapy. In a phase III clinical trial the survival of patients with metastatic CRC was significantly prolonged from 15.6 to 20.3 months when VEGF was neutralized by the antibody bevacizumab.[7] However, it is still an enigma why some patients responded to antiangiogenic therapy and others did not. Biomarkers predicting the response to antiangiogenic therapy are urgently needed to select patients that will most likely benefit from this treatment.[8]

It is consistently documented that chronic inflammation predisposes for CRC[9,10] whereas acute inflammation is regarded as an antitumor defense mechanism.[11] The mechanisms how an acute immune reaction translates into beneficial prognosis are subjects of ongoing research.[12–14] Immune cells can release many different mediators, which can either activate or inhibit angiogenesis, tumor cell proliferation and metastasis and may positively or negatively contribute to tumor cell survival.[3,15–18] In CRC with high T cell density a T-helper 1 (Th-1)-like micromilieu with expression of interferon-γ (IFN-γ) and IFN-γ-induced factors such as IFN regulatory factor-1 (IRF-1), granulysin and granzyme B has been observed and was found to be associated with a positive prognosis.[19,20] This suggested that an IFN-γ-triggered immune response may be beneficial for the patients. It is in agreement with this hypothesis that gene therapeutic approaches with the IFN-γ-induced CXCL9 chemokine suppressed growth and dissemination of colon carcinoma cells in combination with the Th-1-cytokine IL-2 in an *in vivo* model.[21] However, in other reports the expression of indoleamine 2,3-dioxygenase (IDO), a strictly IFN-γ-dependent protein, did not have a significant impact on overall survival of CRC patients[22] and the tumorigenic properties of metastatic CRC cells were described to be fostered by IFN-γ-induced CXCL10 *in vitro*.[23] This demonstrates that further studies are required to determine the impact of IFN-γ-induced genes in the pathogenesis of CRC.

Among the most abundantly induced proteins by IFN-γ is guanylate binding protein-1 (GBP-1).[24–26] GBP-1 belongs to the family of large GTPases which consists of 7 homologous members.[27] We showed that GBP-1 characterizes endothelial cells exposed to IFN-γ, IL-1β and TNF-α, both *in vitro* and *in vivo*[28] and mediates the potent antiangiogenic effects of these cytokines.[29,30] Two different antiangiogenic functions were found to be exerted by GBP-1, first of all the inhibition of endothelial cell proliferation[29] and second, the inhibition of endothelial cell invasiveness.[29] The latter

Additional Supporting Information may be found in the online version of this article.

Abbreviations: CRC, colorectal carcinoma; CXCL9, chemokine (C-X-C motif) ligand 9; CXCL10, chemokine (C-X-C motif) ligand 10; CXCL11, chemokine (C-X-C motif) ligand 11; CXCR3, chemokine (C-X-C motif) receptor 3; EVI, extramural venous invasion; GBP-1, guanylate binding protein-1; IAR, intrinsic angiostatic immune reaction; IC, inflammatory cytokines; IDO, IFN-γ-inducible indoleamine 2,3-dioxygenase; LDAL2, galectin-2; MCP-2, monocyte chemotactic protein-2; OAS2, 2′-5′-oligoadenylate synthetase-2; pM, pathological examined distant metastasis; pN, pathological examined spread into regional lymph nodes; pT, pathological examined size of primary tumor; SLAM, signaling lymphocyte activating molecule; UICC, International Union against Cancer.

Conflict of Interest: E.N., R.S.C. and M.S. declare that parts of the work are subject of a US provisional patent application entitled "Method for the detection of Interferon-associated angiostatic tumorstages in colorectal carcinoma". M.S. has received royalties from a patent for GBP-1 as a target in therapy.

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TABLE I – COLORECTAL CARCINOMA PATIENTS INCLUDED IN AFFYMETRIX ARRAY ANALYSIS (n = 24) AND COLONIC CARCINOMA PATIENTS INCLUDED IN TISSUE ARRAY ANALYSIS (n = 388)

	Tissue array		Affymetrix array	
	n	%	*n*	%
Sex ratio (male/female)	232/156 = 1.5		14/9[1] = 1.55	
Age median / range (years)	64/28–91		63[1]/46–80	
GBP-1 expression in the stroma				
GBP-1-negative (−)	264	68.0	12	50
GBP-1-positive (+)	124	32.0	12	50
Tumor site				
Sigmoid colon	186	47.9	2	8
Descending colon	16	4.1	1	4
Splenic flexure	23	5.9	1	4
Transverse colon	39	10.1	1	4
Hepatic flexure	26	6.7	1	4
Ascending colon	58	14.9	1	4
Cecum	40	10.3	4	17
Rectum			13	54
Stage (UICC 2002)				
I			5	21
II	193	49.7	6	25
III	159	41.0	13	54
IV	36	9.3		
Primary tumor				
pT1			1	4
pT2	27	7.0	6	25
pT3	311	80.2	13	54
pT4	50	12.9	4	17
Regional lymph nodes				
pN0	203	52.3	11	46
pN1	110	28.4	10	42
pN2	75	19.3	3	12
Histopathological grading				
Low grade (G1/G2)	316	81.4	19	79
High grade (G3/G4)	72	18.6	5	21
Extramural venous invasion (EVI)				
EVI (−)	340[2]	87.6	24	100
EVI (+)	46[2]	11.9		
Adjuvant chemotherapy				
No	311	80.2	1	4
Yes	77	19.8	23	96
Emergency presentation				
No	345	88.9	24	100
Yes	43	11.1		

[1]Gender and age of one patient was unknown.–[2]Extramural venous invasion of two patients was unknown.

activity was found to be due to the inhibition of the expression of matrix metalloproteinase-1 and was dependent on the GTPase-activity of the molecule.[30] The inhibition of endothelial cell proliferation was independent of the GTPase activity and specifically mediated by the C-terminal helical domain of the molecule.[29] Recently, we showed that GBP-1 is also secreted from inflammatory cytokine activated endothelial cells, suggesting an extracellular function of the molecule.[31]

The hypothesis of this study was that GBP-1 may be expressed in CRC within a Th-1-like immune reaction and may establish and/or contribute to an intrinsic antiangiogenic antitumor defense reaction in these patients. Therefore, we investigated GBP-1 expression retrospectively in a large cohort of clinically well documented CRC (n = 388) and analyzed the association of this major IFN-γ-induced protein with prognosis of the patients.

Material and methods

Study participants and design

The study is based on specimen and prospectively collected data from well-documented colorectal carcinomas that were undergoing surgery at the Department of Surgery in Erlangen. In a pilot experiment, 10 colorectal carcinomas with the following International Union against Cancer (UICC) stages and tumor sites were included: stage [UICC 2002[32]] I (n = 1), stage II (n = 3), stage III (n = 2) and stage IV (n = 4); rectum (n = 1), sigmoid colon (n = 6), cecum (n = 1), ascending colon (n = 1) and hepatic flexure (n = 1).

In the tissue array, patients undergoing surgery from the year 1991 to 2001 were participating. Median follow-up was 83 months (range 1–177). Patient and tumor characteristics are given in Table I (tissue array). Inclusion criteria of patients in the tissue array were: solitary invasive colon carcinoma (invasion at least of the submucosa) in UICC stage II–IV, localization >16 cm from the anal verge, no appendix carcinoma; no other previous or synchronous malignant tumor, except squamous and basal cell carcinoma of the skin and carcinoma *in situ* of the cervix uteri; carcinoma not arisen in familial adenomatous polyposis, ulcerative colitis or Crohn's disease; treatment by colon resection with formal regional lymph node dissection; residual tumor classification R0 (no residual tumor, clinical and pathohistological examination). Patients who died postoperatively and patients with unknown tumor status (with respect to local and distant recurrence) at the end of the study were excluded. At the end of the study 88 patients (22.7%) had died of their colon carcinoma. Curatively resected distant metastases were located in the liver (n = 29), distant lymph nodes (n = 3), peritoneum (n = 3) and others (n = 3). Metastatic and non-metastatic carcinomas were balanced in the cohort.

Histopathological grading was in accordance with the recommendations of the WHO using the categories low and high grade.[33] With regard to venous invasion we distinguished between no or only intramural venous invasion (EVI negative [−]) and extramural

venous invasion (EVI positive [+]). Emergency presentation was defined as the need for urgent surgery within 48 hr of admission.[34]

For the transcriptome analysis 24 patients who underwent surgery for the first manifestation of CRC were included. Patients who underwent preoperative radiation or chemotherapy did netiher participate in this study nor did patients with familial CRC (familial adenomatous polyposis, hereditary nonpolyposis CRC) or inflammatory bowel disease. UICC stage, sex ratio, patient age, T-, N-, M-category, histopathological grading, tumor site, extramural venous invasion and emergency presentation were used as conventional clinicopathological parameters and were matched as determined by pearson's χ^2 test. Fresh snap frozen biopsies were obtained from all of the patients. Patients were grouped into "GBP-1-negative" ($n = 12$) and "GBP-1-positive" ($n = 12$) according to their GBP-1 expression level as estimated by immunohistochemistry. The patients included in the Affymetrix array analysis ($n = 24$) did not overlap with the patients included in the tissue array collection ($n = 388$, compare upper paragraph) because biopsies had to be taken freshly. All investigations were carried out in accordance with the Helsinki declaration.

Tissue array

Haematoxylin-eosin stained sections of each of the 388 blocks (donor blocks) were evaluated under a microscope. Non-necrotic carcinoma areas in the tumor center, invasive front and of adjacent desmoplastic stroma were identified. Cores were punched of the respective areas in the donor blocks using a tissue micro arrayer (Beecher Instruments, Woodland, USA). A total of 9 punches from each of the 388 blocks were generated originating from tumor center (3 punches), invasive front (3 punches) and desmoplastic stroma (3 punches). Afterwards the cores were embedded (receiver block) and tissue array sections were prepared. In maps of the receiver blocks the exact position of each specimen was documented.

Immunohistochemical staining

Staining for GBP-1, CD31 and CD68 on paraformaldehyde-fixed, paraffin-embedded tissue sections was performed as previously described.[28–30] GBP-1 was detected with a well-established rat monoclonal anti-human GBP-1 antibody [clone 1B1[29–31]]. The mouse anti-human CD31 and CD68 antibodies were purchased from DakoCytomation (Hamburg, Germany). The dilutions were as follows: GBP-1 (enzymatic staining reaction with permanent colour substrate: 1:300/immunofluorescence: 1:20), CD31 (1:50/1:10) and CD68 (1:200/1:20). The fluorochrome-labeled secondary antibodies (goat anti-mouse Alexa 546, goat anti-rat Alexa 488, both Invitrogen, Karlsruhe, Germany) were highly cross-adsorbed to avoid cross-reactivity between mouse and rat. Control stainings were performed (without primary antibody, isotype control) and were negative (data not shown). Stained sections were evaluated by 2 independent persons. Differing results were evaluated by a third person and discussed until the consensus was obtained.

Simultaneous double immunostaining of CD31 and Ki-67 was performed according to Chalmers *et al.*[35] Briefly, CD31 (dilution 1:40) and Ki-67 (DakoCytomation, dilution 1:80) were detected using the EnVision G/2 Doublestain system (DakoCytomation) for simultaneous detection of 2 mouse primary antibodies. Slides were counterstained with Gill-III haematoxylin (Merck, Darmstadt, Germany) and mounted with VectaMount permanent mounting medium (Vector Laboratories, Peterborough, United Kingdom). In each patient microvessel densities and the numbers of proliferating endothelial cells were counted in 3 optical fields (magnification 320×) of the vascular hot spot of GBP-1-positive and -negative areas, respectively. Eight patients from each group (GBP-1-positive and GBP-1-negative) were evaluated in total. The proliferation index was calculated by the relative percentage of proliferating vessels as compared to the total number of vessels for each optical field.

In situ *hybridization*

Biopsy specimens were processed as previously described.[36,37] As a template for transcription of ^{35}S-labeled RNA sense/antisense hybridization probes full length GBP-1-encoding cDNA (gb: M55542) was inserted into the pcDNA3.1 expression vector in sense/antisense orientation. T7 polymerase was used for *in vitro* transcription. After autoradiography sections were stained with haematoxylin and eosin and analyzed in the bright field (expression signals are black silver grains) and dark field (light scattering by the silver grains produces white signals and leads to signal amplification) with a Leica aristoplan microscope (Leica, Solms, Germany) coupled to a Sony CCD live camera (Sony, Munich, Germany).

RT-PCR analysis

RT-PCR analysis was carried out as described by Tripal *et al.*[27] PCR primers (forward/reverse, 5′–3′ orientation) used: GBP-1 (M55542): ATGGCATCAGAGATCCACAT, GCTTATGGTACATGCCTTTC; CXCL10 (NM_001565.1): AAGGATGGACCACACAGAGG, TGGAAGATGGGAAAGGTGAG; CXCL9 (NM_002416.1): TCATCTTGCTGGTTCTGATTG, ACGAGAACG TTGAGATTTTCG; CXCL11 (AF030514.1): GCTATAGCCTT GGCTGTGATAT, GCCTTGCTTGCTTCGATTTGGG; IDO (M34455): GCAAATGCAAGAACGGGACACT, TCAGGGAGACCAGAGCTTTCACAC; MCP-2 (NM_005623): ATTTATT TTCCCCAACCTCC, ACAATGACATTTTGCCGTGA; Mx1 (NM_002462.2): TACAGCTGGCTCCTGAAGGA, CGGCTAA CGGATAAGCAGAG; OAS2 (NM_002535): TTAAATGATAA TCCCAGCCC, AAGATTACTGGCCTCGCTGA; Granzyme A (NM_006144.2): ACCCTACATGGTCCTACTTAG, AAGTGAC CCCTCGGAAAACA; CXCR3-B (AF469635): AGTTCCTGCCAGGCCTTTAC, CAGCAGAAAGAGGAGGCTGT; GAPDH: AGCCACATCGCTCAGAACAC, GAGGCATTGCTGATGATC TTG.

Affymetrix genechip analysis

Affymetrix GeneChip analysis was carried out as described previously.[38–40] The whole microarray experiment design, setup and results are available through ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) using the access number E-MEXP-833.

Statistical analysis

Affymetrix array. Raw data derived from GeneChips were normalized by "global scaling" using Affymetrix Microarray Suite, Data Mining Tool. Signals of the 12 GBP-1-positive and 12 GBP-1-negative CRCs, respectively, were averaged and upregulated genes selected according to $p \leq 0.05$, overall signal intensity >300 relative light units and fold change >4.

Tissue array. The Kaplan-Meier method was used to calculate 5-year rates of cancer-related survival. An event was defined as "cancer-related death," *i.e.* death with recurrent locoregional or distant cancer. The 95% confidence intervals (95% CI) were calculated according to Greenwood.[41] Logrank test was used for comparisons of survival. A Cox regression analysis was performed to identify independent prognostic factors. All factors which were found to be significant in the univariate survival analysis were included in the multivariate model. In the multivariate analysis 2 patients were excluded because of missing data on extramural venous invasion ($n = 386$). χ^2 test was used to compare frequencies. A p-value of less than 0.05 was considered to be statistically significant. Analyses were performed using SPSS software version 13 (SPSS, Chicago, USA).

Proliferation index of vessel. Statistical differences between GBP-1-positive and GBP-1-negative tumors were calculated by student's t-test using SPSS software.

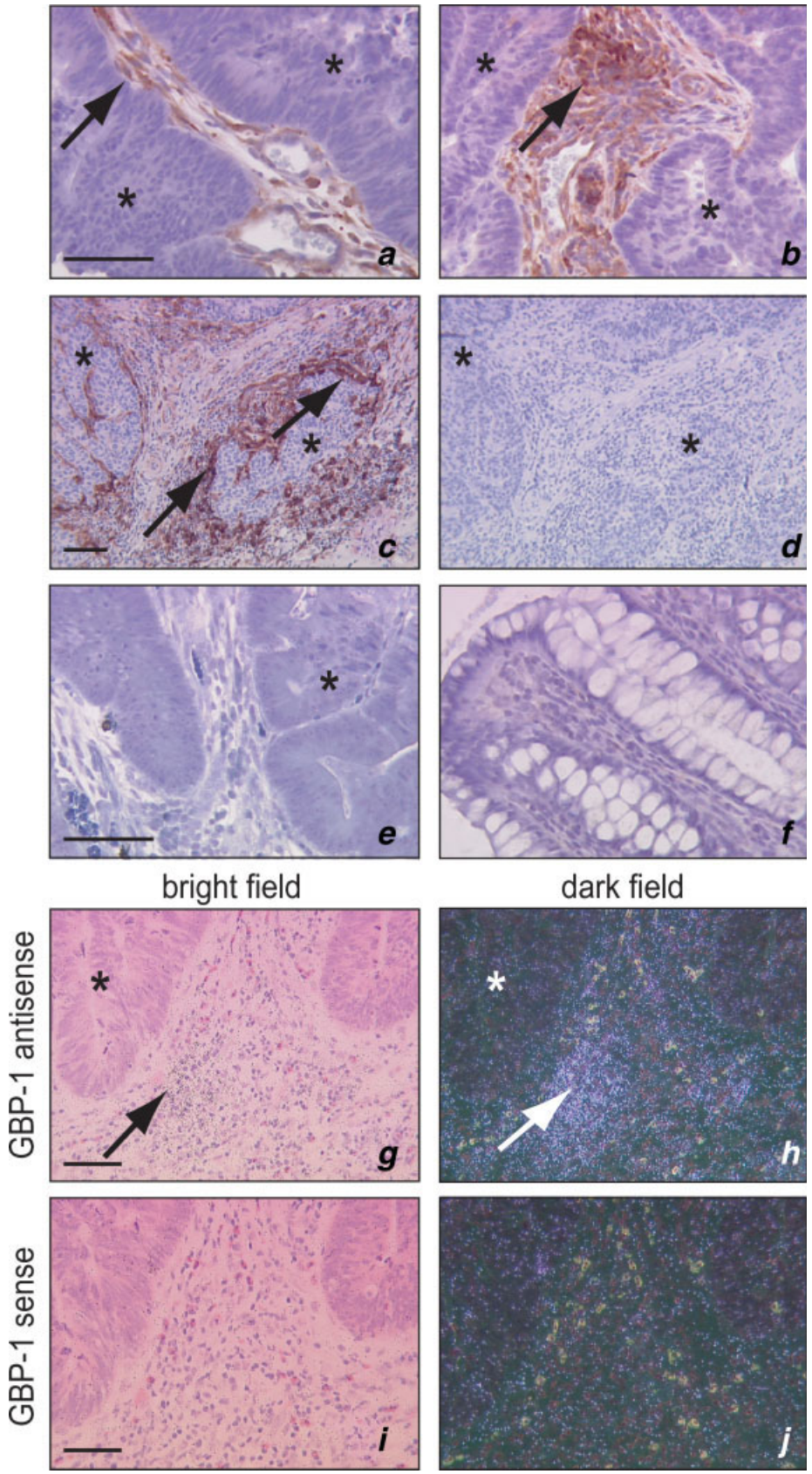

FIGURE 1 – Guanylate binding protein-1 is expressed in stromal endothelial cells and monocytes/macrophages in colorectal carcinoma. Immunohistochemical staining of GBP-1 in (*a–c*, *e*) CRC and (*f*) healthy mucosa tissues of different patients. Examples of GBP-1-positive cells (brown) are indicated by arrows. Areas with tumor cells are labeled by asterisks. (*d*) Negative control of the GBP-1 immunohistochemical staining [without primary antibody, consecutive section of (*c*)]. (*e*) Example of a GBP-1-negative CRC tissue. (*a*, *b*, *e*, *f*) Scale bar corresponds to 50 μm. (*c*, *d*) Scale bar corresponds to 100 μm. Stainings shown in (*a-f*) were carried out with an established rat monoclonal anti-GBP-1 antibody.[28–31] *In situ* hybridization of CRC tissue sections with ^{35}S-radiolabeled GBP-1 (*g*, *h*) antisense and (*i*, *j*) sense RNA strand hybridization probes. Signals were obtained with the antisense hybridization probe (complementary to GBP-1 mRNA) in the stroma of CRC (arrows), both in the (*g*) bright field (black grains) and (*h*) dark field (white grains) exposure. (*i*, *j*) Control hybridization with the GBP-1 sense strand RNA probe did not show specific signals. (*k*) Immunohistochemical staining of GBP-1, CD31 and CD68 by conventional staining on consecutive sections using permanent color substrates. Corresponding tissue areas are indicated by arrows. (*l*) Immunofluorescece double staining and confocal microscopy of GBP-1/CD31 (left panel) and GBP-1/CD68 (right panel) of a GBP-1-positive CRC. (*g–l*) Scale bars correspond to 50 μm.

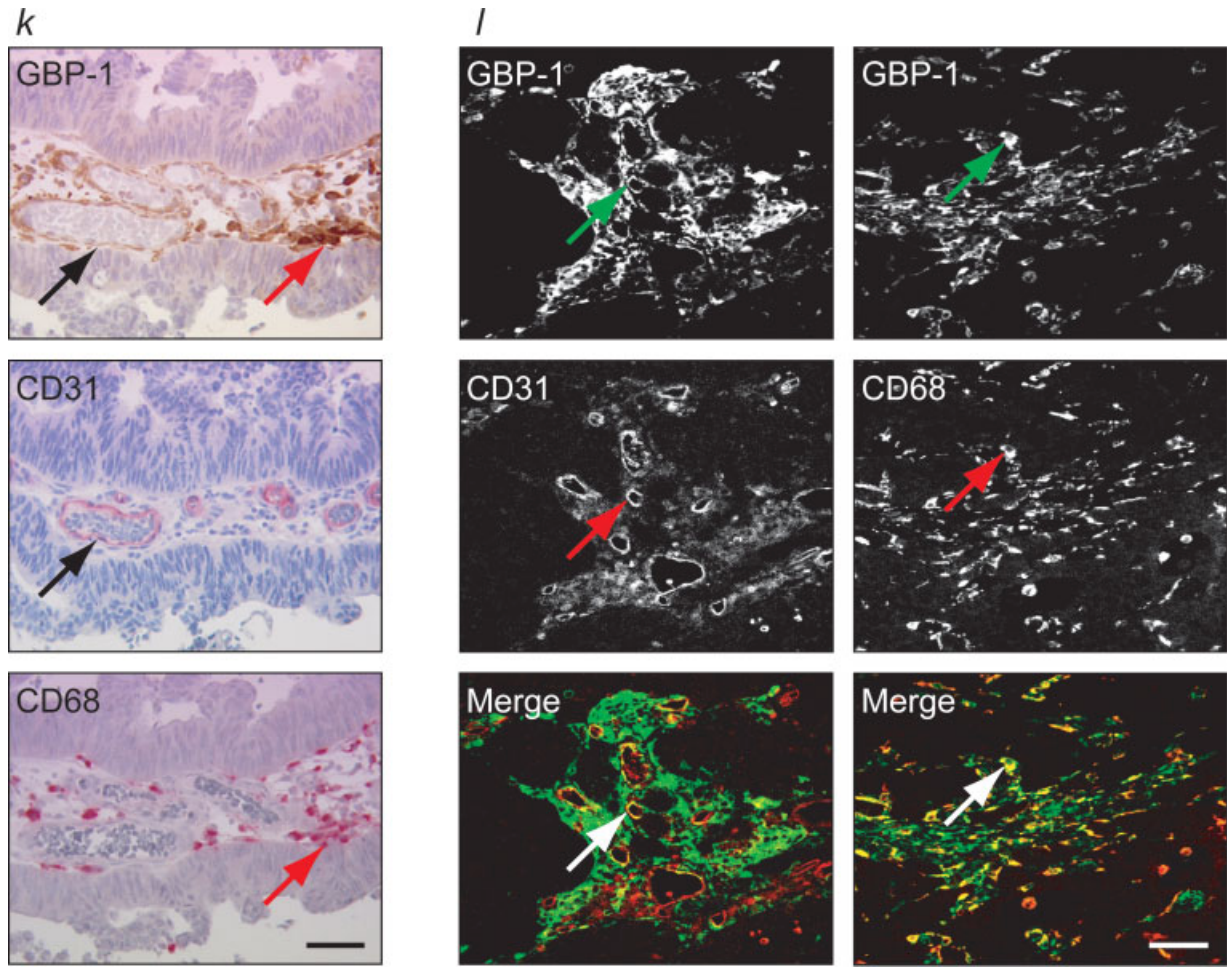

FIGURE 1 – CONTINUED.

Results

GBP-1 is expressed in stromal endothelial cells and monocytes/macrophages in colorectal carcinoma

Robust expression of GBP-1 was detected in a pilot experiment with 10 CRC patients of different UICC stages in 4 cases (Figs. 1*a*–1*c*, arrows) whereas the other 6 tumors were negative (Fig. 1*e*). Consecutive control stainings of GBP-1-positive tumors without primary antibody (Fig. 1*d*, compare Fig. 1*c*) and GBP-1 staining of adjacent tumor-free colon mucosa (Fig. 1*f*) were consistently negative. In GBP-1-positive tumors the protein was selectively expressed in the desmoplastic stroma (Figs. 1*a*–1*c*, arrows) but not in the tumor cells (Figs. 1*a*–1*c*, asterisks). Of note, in sections with high GBP-1 expression the GBP-1-expressing cells were evenly distributed in the desmoplastic stroma of the tumor (Figs. 1*b* and 2*a*, high). In sections with low or intermediate numbers of GBP-1-positive cells the positive cells were located preferentially at the contact sites of tumor cells and desmoplastic stroma cells (Figs. 1*c*, arrows and 2*a*, intermediate).

Immunohistochemical detection of GBP-1 protein in CRC was confirmed by *in situ* hybridization at the RNA level. With a GBP-1 mRNA specific probe strong signals were exclusively obtained in the tumor stroma (Figs. 1*g* and 1*h*, arrows, GBP-1 antisense, bright and dark field of the same tissue section) but not in the tumor cell area (Figs. 1*g* and 1*h*, asterisks). No unspecific signals were obtained when the sense strand negative control probe was used (Figs. 1*i* and 1*j*, GBP-1 sense).

Immunohistochemical staining of GBP-1, CD31 (endothelial cell marker) and CD68 (monocytes/macrophage marker) in consecutive tumor sections demonstrated that GBP-1 is expressed in endothelial cells (Fig. 1*k*, compare black arrows) and monocytes/macrophages (Fig. 1*k*, compare red arrows). These colocalizations were confirmed with a double staining immunofluorescence procedure for simultaneous detection of GBP-1 and CD31 (Fig. 1*l*, left panel, merge, yellow staining, white arrow) or GBP-1 and CD68 (Fig. 1*l*, right panel, merge, yellow staining, white arrow) in GBP-1-positive CRC tissue sections.

GBP-1 expression is associated with early UICC stages and pN0 stage

Subsequently, GBP-1 expression was investigated using the tissue array technology. Formalin-fixed sections of a large cohort of International Union against Cancer (UICC) stage II–IV colonic carcinomas ($n = 388$) with a complete follow-up available were investigated. Non-metastatic (49.7%, UICC stage II) and metastatic (50.3%, UICC stage III and IV) carcinomas were matched in the cohort to prevent metastasis-dependent bias (Table I, tissue array). Other conventional clinical parameters such as sex (male/female = 1.5), age (median 64 years, 28–91 range) or tumor site (*e.g.* 47.9% of all colonic carcinomas in the sigmoid colon) were representative as compared to published statistics of CRC (Table I, tissue array).[42,43] Nine different punches of each patient were analyzed originating from 3 different areas (tumor center, invasive

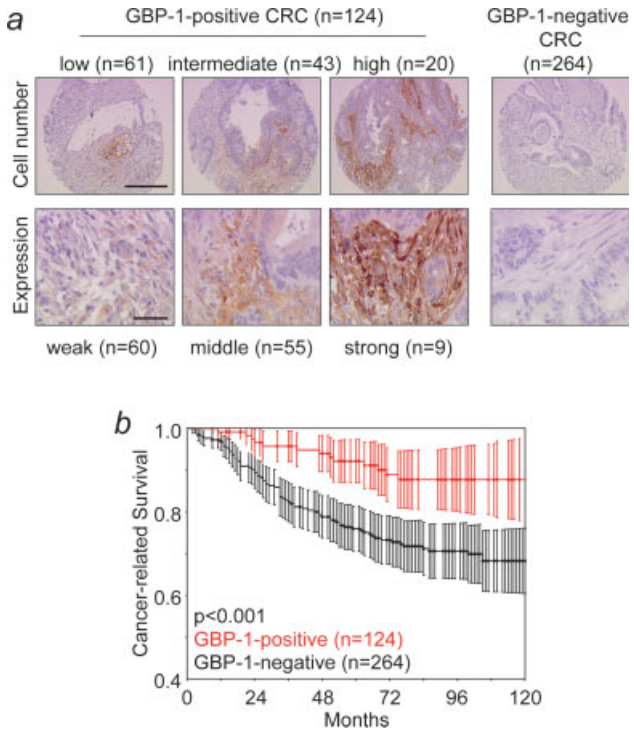

FIGURE 2 – GBP-1 is associated with a prolonged cancer-related 5-year survival in CRC. CRC tissue arrays were immunohistochemically stained for GBP-1 (brown). (*a*) Tissue array punches were grouped into GBP-1-positive and -negative according to the number of GBP-1-positive cells (cell number) and expression strength (expression). Assessment criteria: number of positive cells (negative, low, intermediate and high); expression (negative, weak, middle and strong). The absolute numbers of cases in the different groups are given in brackets. Scale bars correspond to 250 μm (upper panel) and 50 μm (lower panel). (*b*) Cancer-related 5-year survival of patients with GBP-1-positive (red, $n = 124$) and -negative colonic carcinoma (black, $n = 264$). The cancer-related survival is depicted by a Kaplan-Meier-Curve and 95% confidence intervals.

front and desmoplastic stroma) of the carcinoma. The number of GBP-1-positive cells and GBP-1 expression level were estimated and the tumors were categorized accordingly (Fig. 2*a*). GBP-1 was found to be expressed in the desmoplastic stroma of 32% ($n = 124$) of the carcinoma patients whereas 68% were GBP-1-negative ($n = 264$) (Fig. 2*a*, Table II). Comparison of all GBP-1-positive tumors as one group and GBP-1-negative tumors as the other group showed that GBP-1 expression was highly significant ($p < 0.001$) associated with the early tumor stage (Table II, see Stage and Regional Lymph Nodes). In the GBP-1-positive group the majority of tumors were non-metastatic (UICC II: 64.5%; pN0: 67.7%), whereas in the GBP-1-negative group the majority of tumors were metastatic (UICC III + IV: 57.2%; pN1 + pN2: 54.9%) (Table II). In addition, in the GBP-1-positive group the relative numbers of tumors in stage UICC IV and with metastasis in 4 or more regional lymph nodes (pN2) were almost the half (UICC IV: 5.7% *vs.* 11.0%; pN2: 12.1% *vs.* 22.7%) as compared to the GBP-1-negative group (Table II). The described stage-related distributions between both groups were statistically highly significant different ($p < 0.001$, Table II). Other clinical parameters such as primary tumor (pT-classification), histopathological grading or extramural venous invasion did not correlate significantly with GBP-1 expression (Table II).

The same statistical relations were observed when tumors with different levels of GBP-1 expression and numbers of GBP-1-positive cells were compared with the negative tumors (data not shown). Therefore, in the following we did not differentiate between tumors with different expression levels and numbers of positive cells. All GBP-1-positive tumors were grouped together and compared with the GBP-1-negative tumors (Fig. 2*a*).

TABLE II – GBP-1 EXPRESSION IS HIGHLY SIGNIFICANT ASSOCIATED WITH EARLY UICC STAGE II/PN0-STATUS OF COLONIC CARCINOMA ($n = 388$)

	GBP-1 positive	GBP-1 negative	*p* value
N	124 (32.0%)	264 (68.0%)	
Stage (UICC 2002)			<0.001
II	80 (64.5%)	113 (42.8%)	
III	37 (29.8%)	122 (46.2%)	
IV	7 (5.7%)	29 (11.0%)	
Primary tumor			0.411
pT2	11 (8.9%)	16 (6.1%)	
pT3	100 (80.6%)	211 (79.9%)	
pT4	13 (10.5%)	37 (14.0%)	
Regional lymph nodes			<0.001
pN0	84 (67.7%)	119 (45.1%)	
pN1	25 (20.2%)	85 (32.2%)	
pN2	15 (12.1%)	60 (22.7%)	
Histopathological grading			0.264
Low grade (G1/G2)	97 (78.2%)	219 (83.0%)	
High grade (G3/G4)	27 (21.8%)	45 (17.0%)	
Extramural venous invasion			0.056
EVI (−)	114[1] (92.7%)	226[1] (85.9%)	
EVI (+)	9[1] (7.3%)	37[1] (14.1%)	

[1]Extramural venous invasion of two patients was unknown. *p* Value was determined by Pearson's χ^2 test.

GBP-1 is associated with a prolonged cancer-related 5-year survival and a halved risk of cancer-related death

The patients with GBP-1-positive colonic carcinoma had a highly significant ($p < 0.001$) increased cancer-related 5-year survival rate of absolutely 16.2% in univariate analysis (Table III, univariate analysis, Fig. 2*b*). The cancer-related 5-year survival rate increased from 76% in the GBP-1-negative patients up to 92.2% in the GBP-1-positive patients (Table III, univariate analysis, GBP-1 expression). Other well-established prognostic factors such as UICC stage, pT- and pN-category or extramural venous invasion did correlate with increased survival confirming the representative value of this study group (Table III, univariate analysis).

Finally, we investigated whether GBP-1 expression indicated increased survival in all tumor stages or was associated with increased survival because of its preferential expression in early stages. To this goal a multivariate cox regression analysis was carried out. This analysis revealed that GBP-1 expression is an independent prognostic factor ($p = 0.032$) indicating a reduction of the relative risk of cancer-related death by the half (Table III, multivariate analysis, GBP-1 expression). Conventional clinical parameters such as UICC stage or extramural venous invasion reported as independent prognostic factors showed a highly significant association in the study group, confirming the representative value of the patient's collection.

GBP-1 expression is associated with a Th-1-like immune reaction and decreased angiogenic activity

To characterize in more detail the microenvironment associated with GBP-1 a transcriptome analysis was carried out. Freshly snap frozen biopsies of CRC patients were immunohistochemically analyzed for GBP-1 expression and 12 GBP-1-positive *versus* 12 GBP-1-negative CRC patients with closely matched clinical parameters (Table I, Affymetrix array) were selected. RNA of these 24 CRC patients was subjected to a transcriptome analysis (HG-U133A, Affymetrix, 22,215 probe sets). Gene expression results were sorted according to their probability of differential expression ($p < 0.05$) between both groups, significant signal intensity

TABLE III – CANCER-RELATED SURVIVAL: UNIVARIATE ANALYSIS (n = 388) AND MULTIVARIATE COX REGRESSION ANALYSIS (n = 386)

	Univariate analysis				Multivariate analysis			
	n	5-year cancer-related survival (%)	95% CI	p value	n	Relative risk	95% CI	p value
All patients	388	81.1	77.2–85.0		386[1]			
GBP-1 expression in the stroma				<0.001				
GBP-1-negative (−)	264	76.0	70.7–81.3		263	1.0		
GBP-1-positive (+)	124	92.2	87.3–97.1		123	0.5	0.3–0.9	0.032
Stage (UICC 2002)				<0.001				
II	193	91.6	87.5–95.7		193	1.0		
III	159	74.2	67.3–81.1		157	2.5	1.5–4.2	0.001
IV	36	57.3	40.8–73.8		36	4.3	2.2–8.3	<0.001
Primary tumor				0.005				
pT2	27	96.2	88.8–100					
pT3	311	82.3	78.0–86.6					
pT4	50	64.8	51.3–78.3					
Regional lymph nodes				<0.001				
pN0	203	90.0	85.7–94.3					
pN1	110	86.2	79.7–92.7					
pN2	75	49.1	37.3–60.9					
Histopathological grading				0.134				
Low grade (G1/G2)	316	82.4	78.1–86.7					
High grade (G3/G4)	72	75.2	65.0–85.4					
Extramural venous invasion				<0.001				
EVI (−)	340[1]	85.8	82.1–89.5		340	1.0		
EVI (+)	46[1]	47.6	32.7–62.5		46	2.7	1.7–4.4	<0.001
Adjuvant chemotherapy				0.207				
No	311	82.4	78.1–86.7					
Yes	77	75.7	65.9–85.5					
Emergency presentation				<0.001				
No	345	83.7	79.8–87.6		344	1.0		
Yes	43	57.8	42.1–73.5		42	2.1	1.2–3.7	0.008

[1]Extramural venous invasion of two patients was unknown.

(>300 RLUs) and robust upregulation of expression (>4-fold) in the GBP-1-positive tumors. 104 genes fulfilled these criteria (Supplementary Table I). Most of these genes were well-known IFN-induced genes, chemokines and immune reaction-associated genes (Supplementary Table I). Interestingly, the 3 major angiostatic chemokines (CXCL9, CXCL10, CXCL11: supplementary Table I, shaded)[44,45] were among the 8 most strongly upregulated genes in GBP-1-positive tumors. The expression of angiogenic growth factors such as VEGF and basic fibroblast growth factor (bFGF) was not increased in GBP-1-positive CRC.

High reproducibility of the microarray analyses is demonstrated by the fact that within the groups of GBP-1-positive and -negative tumors highly reproducible results were obtained for each gene as shown exemplarily for GBP-1, CXCL9 and CXCL11 (Fig. 3*a*). In addition, semi-quantitative RT-PCR confirmed the microarray results showing that each of the 3 angiostatic chemokines (CXCL10, CXCL9, CXCL11) and of 5 additional IFN-γ-induced and/or immune reaction-associated genes [IFN-γ-inducible indoleamine 2,3-dioxygenase (IDO), monocyte chemotactic protein-2 (MCP-2), Mx1, 2′-5′-oligoadenylate synthetase-2 (OAS2) and granzyme A] were higher expressed in 3 GBP-1-positive as compared to 3 GBP-1-negative tumors (Fig. 3*b*).

The antiangiogenic chemokines CXCL9-11 inhibit angiogenesis selectively *via* the chemokine receptor CXCR3-B.[46,47] RT-PCR showed that this receptor is constitutively expressed in both, GBP-1-positive and -negative CRC (Fig. 3*c*, CXCR3-B). Thereby, the molecular precondition that CXCL9-11 may act angiostatic in the corresponding CRC was found to be fulfilled. Finally, immunohistochemical investigation of 8 GBP-1-positive and 8 GBP-1-negative CRC showed that proliferating (Ki-67-positive) endothelial cells (CD31-positive) are present in highly significant increased numbers in GBP-1-negative vessels (Fig. 3*d*, upper panel, black arrowheads) as compared to GBP-1-positive vessels (Fig. 3*d*, lower panel, open arrowheads). The average proliferation index (Ki-67-positive vessels per total vessels) was 2.21% ± 1.91% in GBP-1-positive CRC as compared to 6.64% ± 5.69% in GBP-1-negative CRC (Fig. 3*e*, $p < 0.001$). This confirmed that angiogenic activity is reduced in GBP-1-expressing CRC.

Discussion

We showed in previous work that inflammatory cytokines including IFN-γ induce the expression of GBP-1 in vascular endothelial cells.[27–29] GBP-1 was found to inhibit endothelial cell proliferation and invasion and to mediate the angiostatic effects of inflammatory cytokines on endothelial cells *via* these activities *in vitro*.[29,30] In agreement with this, GBP-1 expression was demonstrated to be associated with inflammatory reactions and with decreased angiogenic activity of vascular endothelial cells *in vivo*.[28,29] Specifically, this has been demonstrated in Kaposi's sarcoma (KS) resulting in the conclusion that angiogenic activation driven by bFGF and VEGF and inflammatory angiostatic processes are present in KS.[29]

Also CRC is characterized by the pathogenic interplay of inflammation and angiogenesis, which directed us to characterize the expression and function of GBP-1 in this disease. We could show that GBP-1 is expressed in 32% of the patients suffering from CRC. GBP-1-expression was associated with a highly significant increased cancer-related 5-year survival rate of absolutely 16.2%. Most importantly, multivariate analysis demonstrated that GBP-1 is an independent prognostic factor indicating a halved risk of cancer-related death.

When analyzing GBP-1-associated gene expression as an indicator of the tumor microenvironment we found that 90 of the 103 coregulated genes upregulated encoded immunoglobulins, immune cell receptors or other molecules with important functions in immune reactions. For example, signaling lymphocyte activating molecule (SLAM, 7.45-fold upregulated in GBP-1-positive CRC) and CD38 (4.25-fold) are involved in the regulation of immune response.[48–50] Another example, galectin-2 (LDAL2, 4.21-fold), triggers inflammation of myocardial infection.[51] Strikingly, 23 of the 90 genes encoded major IFN-γ-induced factors such as IDO (8.51-fold), OAS2 (5.25-fold), IFI30 (4.51-fold) and Mx1 (4.36-fold). Most importantly, 3 of the 4 presently known antiangiogenic chemokines, namely CXCL9 (14.36-fold), CXCL10 (13.01-fold) and CXCL11 (25.52-fold) were very highly upregulated in GBP-1-positive tumors. The high significance of

the transcriptome analysis was demonstrated by the fact that almost all (90 of 103) GBP-1-coexpressed genes supported congruently the biologically meaningful conclusion that GBP-1 is associated with a potent IFN-γ-dominated Th1-like immune reaction with potential angiostatic activity.

Of note, the human GBP family presently consists of 7 members.[27] In the GeneChip analysis only GBP-1 and GBP-2 were included as target genes. Therefore, we investigated the expression of the other GBPs by semi-quantitative RT-PCR. This study showed that also GBP-2 to -5 were upregulated in GBP-1-positive CRC, whereas GBP-6 and GBP-7 were not expressed (data not shown). Interestingly, the expression of GBP-1 to -3 is known to be induced by IFN-γ, TNF-α and IL-1β, whereas the expression of GBP-4 and GBP-5 is selectively induced by IFN-γ.[27] The latter supported that the microenvironment in GBP-1-positive CRC is dominated by IFN-γ.

The clinical impact of a Th-1-like immune reaction in CRC was still a matter of discussion until recently. Clinical trials of adjuvant therapy with IFN-γ did not show clinically meaningful benefit[52] and *in vitro* studies showed that for example expression of strictly IFN-γ-dependent proteins such as indoleamine 2,3-dioxygenase (IDO) reduced antitumoral T cell attack in a murine model[53] or that CXCL10 fostered tumorigenic properties of metastatic colorectal cells.[23] However, in CRC patients IDO expression was not associated with a significant impact on overall survival[22] and CXCL10 expression and its effect on patient's survival were not investigated in the CRC to our knowledge. Only recently, a very carefully conducted study with 3 independent patient cohorts showed that Th-1 adaptive immunity with high CD3 T cell density and expression of IFN-γ and of IFN-γ-induced genes was of clear benefit for the patients and may be even a better predictor of increased survival and decreased recurrence of patients with CRC as the conventional TNM-classification.[19,20,54] In addition, intratumoral expression of Th-1 cytokines has been described to be high in benign adenoma and to decrease in progressed stages of CRC.[55] Our findings are in clear agreement with the 2 latter studies supporting a Th-1-like IFN-γ-dominated reaction that may counteract tumor progression. Beyond this, we show that numerous angiostatic factors including GBP-1 and the antiangiogenic chemokines CXCL9-11 are associated with this immunoreaction in CRC. This indicated that the beneficial effect of this reaction on patient's survival may not only be due to a specific cytotoxic immunoreaction directed against the tumor cells but also due to a potent antiangiogenic micromilieu. The high sensitivity of CRC to the inhibition of angiogenesis is clearly documented by the fact that CRC was the first human cancer where antiangiogenic therapy was successfully applied.[7]

These findings may provide a new perspective for the understanding of the mechanisms of IFN-γ-mediated tumor rejection, which are still under investigation.[56] For example, IFN-γ can act directly on the tumor cells. It increases MHC class I and II expression,[57,58] inhibits cell proliferation[59] and suppresses c-myc expression[60] in the tumor cells. Currently evidence increases that IFN-γ can induce CRC rejection by acting on the stroma cells.[56] In this framework, the angiostatic activity of IFN-γ is regarded to be the most important effect. Potential angiostatic effects of IFN-γ are the induction of the expression of angiostatic chemokines[61,62] and the suppression of VEGF-A expression in monocytes by posttranscriptional mechanisms.[12] In addition, it is accepted that IFN-γ inhibits angiogenic activity of blood and lymph vessel endothelial cells *in vitro*.[29,63] However, it is still under debate whether *in vivo*

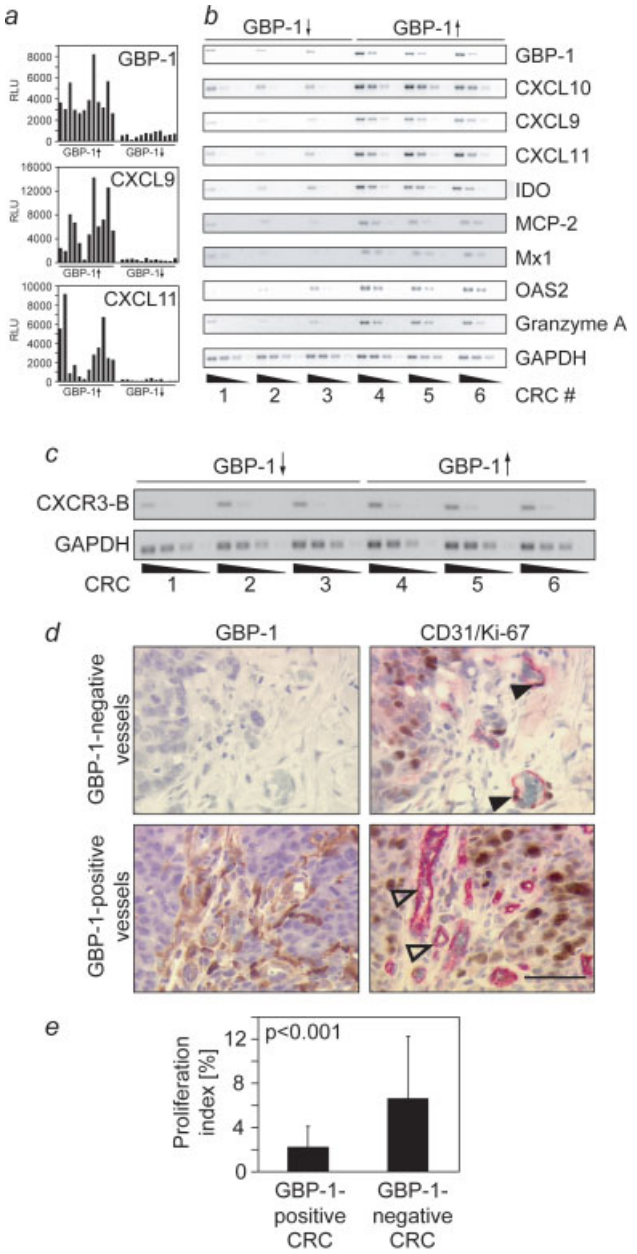

FIGURE 3 – GBP-1 expression is associated with a Th-1-like immune reaction and decreased angiogenic activity. (*a*) Normalized microarray signal intensities (relative light units: RLU) of GBP-1, CXCL9 and CXCL11 expression in GBP-1-positive (GBP-1↑, $n = 12$) and GBP-1-negative CRC (GBP-1↓, $n = 12$). The tumors are given at corresponding positions in each diagram. (*b*) Semi-quantitative RT-PCR of GBP-1-coregulated genes (CXCL10, CXCL9, CXCL11, IDO, MCP-2, Mx1, OAS2 and granzyme A) in 3 different GBP-1-positive (GBP-1↑) and GBP-1-negative (GBP-1↓) CRC. Decreasing amounts of cDNA (undiluted, 1/10, 1/100 and 1/1,000) of the different tumors were subjected to each PCR. Amplification of GAPDH demonstrates that equal amounts of cDNA were used from each tumor. (*c*) CXCR3-B expression was analyzed with semi-quantitative RT-PCR in 3 GBP-1-positive (GBP-1↑) and GBP-1-negative (GBP-1↓) CRC. cDNA was subjected in decreasing amounts (undiluted, 1/10, 1/100 and 1/1,000) to the PCR. Amplification of GAPDH demonstrates that equal amounts of cDNA of the different tumors were used. (*d*) Immunohistochemical staining of GBP-1 (left) and simultaneous double staining (right) of CD31 (red) and Ki-67 (proliferation-associated antigen, brown) on consecutive sections of GBP-1-negative (upper panel) and -positive (lower panel) CRC. Proliferating vessels are indicated by black arrowheads, nonproliferating vessels by open arrowheads. Scale bar corresponds to 50 μm. (*e*) Proliferation index [%] of GBP-1-positive and -negative CRC as estimated by quantification of Ki-67/CD31-positive vessels in comparison to the total vessel number of the same optical field. Three optical fields in the hot spot of each section were counted (GBP-1-positive: $n = 8$; GBP-1-negative: $n = 8$).

inhibition of angiogenesis is mediated by indirect or direct effects of IFN-γ on tumor vessel endothelial cells.[13,14]

Indirect effects of IFN-γ may target tumor cells and nonendothelial stroma cells inducing in these cells the release of angiostatic cytokines/chemokines or inhibiting VEGF expression. Alternatively, IFN-γ may inhibit angiogenesis by direct inhibition of endothelial cell proliferation, migration and invasion. The results described here clearly support that not only indirect but also direct effects are present in the CRC. First of all, GBP-1 expression itself demonstrated that endothelial cells and monocytes are directly exposed to and are functionally activated by Th-1 cytokines such as IFN-γ. Of note, in tumors with low or intermediate numbers of positive cells GBP-1 expression was preferentially detected in endothelial cells in the close proximity of tumor cells (Figs. 1*c*, arrows, and 2*a*). This is well in accord with the fact that IFN-γ expression in T cells is tightly regulated and requires antigen recognition.[56] Accordingly, IFN-γ should first induce GBP-1 expression in those endothelial cells which are closest to the interaction site of tumor cells and T cells. Second, we detected a strong association of GBP-1 with CXCL9, CXCL10 and CXCL11 expression. These chemokines are members of the CXC chemokine family lacking the conserved "ELR-motif", which have been shown to be potent inhibitors of angiogenesis.[64] Their common receptor CXCR3 is present in 2 alternative splice variants. Of these only one form (CXCR3-B) mediates the antiangiogenic effects of the chemokines.[46] Interestingly, CXCR3-B receptor was expressed in CRC tissues (Fig. 3*c*). Therefore, the chemokines CXCL9-11 may act antiangiogenic in this microenvironment.

Altogether *in vivo* 2 effects have to be considered: (*i*) direct angiostatic effects of Th-1-associated cytokines on endothelial cells mediated by GBP-1 in CRC tissues and (*ii*) indirect angiostatic effects *via* the induction of CXCL9-11 expression, which may further amplify the inhibition of EC proliferation.

A perspective for the implementation of our results in the clincial treatment of CRC is based on the hypothesis that it is very likely that patients will respond differentially to antiangiogenic treatment. In consequence, it will be interesting to evaluate GBP-1 expression retrospectively in clinical studies using antiangiogenic treatment to analyze whether GBP-1 expression may be associated with a specific therapy response.

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