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Macrophage migration inhibitory factor (MIF) expression in human glioblastomas correlates with vascular endothelial growth factor (VEGF) expression

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Abstract

Macrophage migration inhibitory factor (MIF) is a peptide released upon hypothalamo-pituitary stimulation that acts as a potent endogenous antagonist of the glucocorticoid inhibition of acute inflammatory response and subsequent antigen-specific response. MIF also sustains tumour growth as it promotes angiogenesis, overcomes p53-mediated cell growth arrest and inhibits tumour-specific immune responses. Using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry we studied MIF expression in 35 human glioblastomas and two normal brains. We compared these results with the expression of vascular endothelial growth factor (VEGF), the most potent angiogenic factor in glioblastomas. We detected MIF in normal cortical neurons and glial cells. All glioblastomas were positive for MIF mRNA with expression levels similar to or higher than those of normal brain. MIF immunoreactivity was seen mainly in tumour cells and less frequently in hyperplastic endothelial cells. The expressions of MIF and VEGF mRNA were strongly correlated ($P < 0.0001$). Our results demonstrate the expression of MIF in human glioblastomas, and indicate a close relationship with VEGF expression. This is of particular interest given the potential modulation of MIF by glucocorticosteroids.

Keywords: MIF; glioblastoma; brain; tumour; VEGF

Introduction

Looking at the differential expression of an array of 6000 genes in the normal brain and in four glioblastomas (GBMs), Markert *et al.* [14] reported a specific upregulation of the macrophage migration inhibitory factor (MIF) in 3/4 GBMs. It has been hypothesized that MIF sustains tumour growth by multiple mechanisms. MIF inhibits p53 suppressor activity *in vitro* [9], which could be relevant in astrocytic gliomas where the loss of p53 tumour suppressor activity is a common finding [11]. MIF also shows angiogenic activity both *in vitro* [22] and *in vivo* [6]. Interestingly, GBMs display a prominent angiogenesis and endothelial hyperplasia. In addition, MIF acts as the most potent endogenous antagonist of glucocorticosteroid at both a local and a systemic level (for a review, see Metz *et al.* [16]), while MIF secretion and production are both modulated by exogenous corticosteroids. These properties might affect the response of GBMs to glucocorticosteroid therapy, currently used in the management of GBMs.

We therefore studied the expression of MIF in a series of 35 human GBMs and in two normal brains using immunohistochemistry in all cases and reverse transcriptase polymerase chain reaction (RT-PCR) in 22 cases for which frozen tissue was available.

We further compared MIF expression with the expression of the isoforms of the vascular endothelial growth factor (VEGF), known for its angiogenic role in GBMs [13].

Materials and methods

Patient selection

We investigated 35 histologically proven GBMs examined at the Laboratory of Neuropathology CHU Liège, between 1997 and 2001. The series included 29 primary GBMs (i.e. no previous history of lower grade diffuse astrocytoma) and six secondary GBMs (i.e. previous history of lower grade diffuse astrocytoma). The sex ratio was 18F/17M, and the age at time of diagnosis ranged from 32 to 79 years with a mean value of 53 years. At time of surgery all patients were given solumedrol 125 mg intravenously. There was no recent prior history of corticotherapy. Immunostaining for p53 was performed in 32 cases: positive staining, defined as the presence of at least 10% positive nuclei in 10 consecutive high power (400×) tumour fields, was present in 17/32. Normal brain cortex and white matter were obtained from two specimens of temporal lobes resected for intractable epilepsy. This study was approved by the Ethical Committee of the Faculty of Medicine of the University of Liège.

RNA extraction and cDNA synthesis

Total RNA was extracted from cryosections with the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed with a ThermoScript reverse transcriptase (ThermoScript™ RT-PCR System, Invitrogen, Carlsbad, CA, USA) and random hexamer as primer.

Primers

Primers (Table 1) for the VEGF gene (GenBank accession no. AH001553) were chosen to distinguish $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$ mRNA isoforms. Primers for the human MIF-1 gene (GenBank accession no. NM_002415) were chosen using the primer design software Primer Express™ (Perkin-Elmer Applied Biosystems, Branchburg, New Jersey, USA). We conducted BLASTn (National Center for Biotechnology Information, Bethesda, MA, USA) searches against dbEST and the nonredundant set of GenBank, EMBL, and DDBJ database sequences to confirm the total gene specificity of the nucleotide sequences chosen for the primers. The 18S ribosomal RNA was measured using the Pre-Developed TaqMan Assay Reagents Endogenous control kit from Applied Biosystems.

Table 1 *Primers used in PCR analysis*

Gene	*Accession no.*	*Primers*	*Position*
MIF-1	NM_002415	5'-GAACCGCTCCTACAGCAAGCT-3' (forward primer)	313-441
		5'-GCGAAGGTGGAGTTGTTCCA-3' (reverse primer)	
VEGF	AH001553	5'-CCTGGTGGACATCTTCCAGGAGTA-3' (forward primer)	1208-1687
		5'-CTCACCGCCTCGGCTTGTCACA-3' (reverse primer)	

Quantitative end-point PCR for VEGF mRNA isoforms

$VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$ mRNA isoforms were measured in 10 ng aliquots of cDNA using Taq polymerase (Takara, Shigu, Japan) and 5 pmol of each primer (Table 1). The thermal cycling conditions included 2 min at 95°C for denaturation and then amplification for 15 s at 94°C, 20s at 66°C and 30 s at 72°C (33 cycles for VEGF isoforms) with a final incubation of 2 min at 72°C. PCR products were resolved on 2% Nusieve 3:1 agarose gels (BioWhittaker, Rockland, USA) and analysed using a Fluor-S Multilmager (Bio-Rad, Hercule, CA, USA) after ethidium bromide staining. The expected sizes for $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{120}$ were, respectively, 479, 407, 347 and 275 bp. VEGF mRNA levels were expressed as the ratio of VEGF transcripts to 18S transcripts. The experiments were repeated at least three times in duplicate.

Real-time quantitative PCR

The quantification of the starting amount of the MIF mRNA in an unknown sample was performed by preparing a standard curve using known dilutions of a cDNA. PCR amplification was performed using the SYBR Green PCR master mix for MIF mRNA or the TaqMan universal PCR master mix and the Pre-Developed TaqMan Assay Reagents Endogenous Control kit for 18S rRNA (PE Applied Biosystems) according to the manufacturer's protocol. The thermal cycling conditions included 2 min at 50°C, 10 min at 95°C and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C. The ABI PRISM 7700 Sequence Detection System instrument (PE Applied Biosystems) was used to measure fluorescence in real time. To confirm amplification specificity, the PCR products were also examined by subsequent 2% agarose gel electrophoresis.

For each experimental sample, the amounts of the MIF and 18S rRNA were determined from the calibration curve. The target amount was then divided by 18S rRNA to obtain a normalized target value (as described by the manufacturer PE Applied Biosystems in User Bulletin no. 2). The experiments were repeated at least three times in duplicate.

Immunohistochemistry for MIF and VEGF

Sections (thickness 4 μm) were cut from formalin-fixed, paraffin-embedded tumour tissue. They were hydrated through graded alcohols, incubated in H_2O_2 (0.3%, 15 min) and blocked in normal rabbit serum (1:10, 1 h, Vector, Burlingame, CA, USA). Sections were autoclaved for 11 min at 126°C in citrate buffer pH 6 for antigen retrieval. They were incubated in primary polyclonal antibody (Ab) anti-MIF 1:250 (R&D, Minneapolis, MN, USA) or anti-VEGF 1:150 (Santa Cruz, Santa Cruz, CA, USA) for 1 h at room temperature, followed by secondary Ab (1 h) and avidin-linked horseradish peroxidase (30 min, Dako, Glostrup, Denmark). Immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB+, Dako). Negative controls were obtained by omitting the primary antibody. Samples of human anterior pituitary obtained from *post-mortem* cases were

used as positive controls for MIF: they showed a characteristic mosaic pattern due to selective staining of corticotrophin (ACTH)- and thyrotrophin (TSH)-producing cells in accordance with previous reports (Figure 1a) [17,23]. In tumour specimens, the immunopositivity was assessed semiquantitatively, based on the density of positive cells with 0 = complete absence, 1 = rare strongly positive cells, 2 = moderate density of positive cells, and 3 = strong diffuse positivity.

Statistics

Given the relatively small sample size, we chose nonparametric tests for correlation studies. We used Spearman's test to look for correlation between MIF mRNA, VEGF mRNA and age of the patients. Samples were further grouped according to results of MIF, VEGF and p53 immunohistochemistry and tumour subtypes. Correlation between groups was assessed using Mann-Whitney and Kruskal-Wallis tests. Two-tailed *P*-values were considered significant at <0.05. Statistical analyses were performed using prism 3.0 software (GraphPad, San Diego, CA, USA).

Results

Expression of MIF

RT-PCR showed a basal expression of MIF mRNA in both of the samples of normal brain (Figure 2). By immunohistochemistry, MIF was detected in the cortex in some pyramidal neurons and in a few glial cells, showing strong cytoplasmic positivity. A weaker diffuse staining was also seen in the cortical neuropile (Figure 1b).

MIF expression was seen in all GBMs, with variable intensity: in 5/20 cases mRNA expression was at least twice as high as in the normal brain; the other tumours showed mRNA levels in the range of normal brain values. Immunostaining for MIF was mainly seen in tumour cells with a frequent additional staining of hyperplastic endothelial cells in proliferating capillaries (Figure 1c). We found no correlation between the scores of MIF immunostaining (Table 2) and the level of mRNA expression in the tumours ($P = 0.3841$, Kruskal-Wallis test). Interestingly, a strong immunopositivity for MIF was also detected in reactive astrocytes scattered in the surrounding peritumoral oedema (Figure 1d). No correlation was found between the levels of MIF mRNA and the subtype of glioblastoma ($P = 0.1689$, Mann-Whitney test), the age of patients ($P = 0.6451$, Spearman test) and p53 immunostaining status ($P = 0.1388$, Mann-Whitney test).

Expression of VEGF

Expression of VEGF mRNA was minimal in normal brain (Figure 3). By immunohistochemistry, VEGF peptide was localized to the endothelium of rare capillaries in the white matter (Figure 1f). In GBMs, the expression of VEGF mRNA was highly variable, ranging from values similar to normal brain to a 15-fold increase (Figure 3a,b): 14/20 (70%) cases showed mRNA levels at least two times those of normal brain values. By immunohistochemistry 29/35 (83%) cases showed a positive staining of hyperplastic endothelium and/or tumour cells (Figure le). The immunostaining was generally stronger on endothelial cells than on tumour cells. When present in specimens, VEGF positivity was diffuse and there was no spatial restriction to perinecrotic areas. There was no labelling of peritumoral reactive astrocytes. We found no correlation between the intensity of VEGF immunopositivity (Table 2) and the level of VEGF mRNA ($P = 0.5478$, Kruskal-Wallis test), age of patients ($P = 0.2995$, Spearman test), tumour subtype ($P = 0.3971$, Mann-Whitney test), and p53 immunostatus ($P = 0.1672$, Mann-Whitney test).

The expression pattern of VEGF mRNA isoforms was similar in normal brain and GBMs, with a predominant representation of the $VEGF_{165}$ and $VEGF_{121}$ isoforms (Figure 3 c).

Correlation between MIF and VEGF

Using quantitative RT-PCR, we showed a strong correlation ($P< 0.0001$, Spearman test) between the expression of MIF and VEGF mRNAs (Figure 4). Interestingly, the scores for MIF and VEGF immunostaining correlated significantly ($P = 0.0329$, Kruskal-Wallis test). Although no double immunostaining was performed for MIF and VEGF, examination of serial sections suggested in several cases a strong coexpression of MIF and VEGF by tumour cells. We found no correlation between MIF immunohistochemistry and VEGF mRNA levels ($P = 0.4107$, Kruskal-Wallis test), or between VEGF immunohistochemistry and MIF mRNAlevels ($P = 0.1548$, Kruskal-Wallis test).

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***Figure 1**. (a-d) MIF immunostaining, Original magnification × 400. (a) Mosaic staining in normal human anterior pituitary. (b) Normal brain cortex showing positive staining in the perikaryon of some pyramidal neurons (arrows) and in possible glial cells (open arrow). (c) Glioblastoma: a strong staining is seen in the cytoplasm of tumour cells and hyperplastic endothelium (arrow). (d) Staining of reactive astrocytes in the peritumoral oedema. (e,f) VEGF immunostaining, Original magnification × 400. The strong positivity of tumour cells and hyperplastic endothelium (arrow) in GBM (e) contrasts with the staining of flat endothelial cells in rare capillaries of normal white matter (f).*

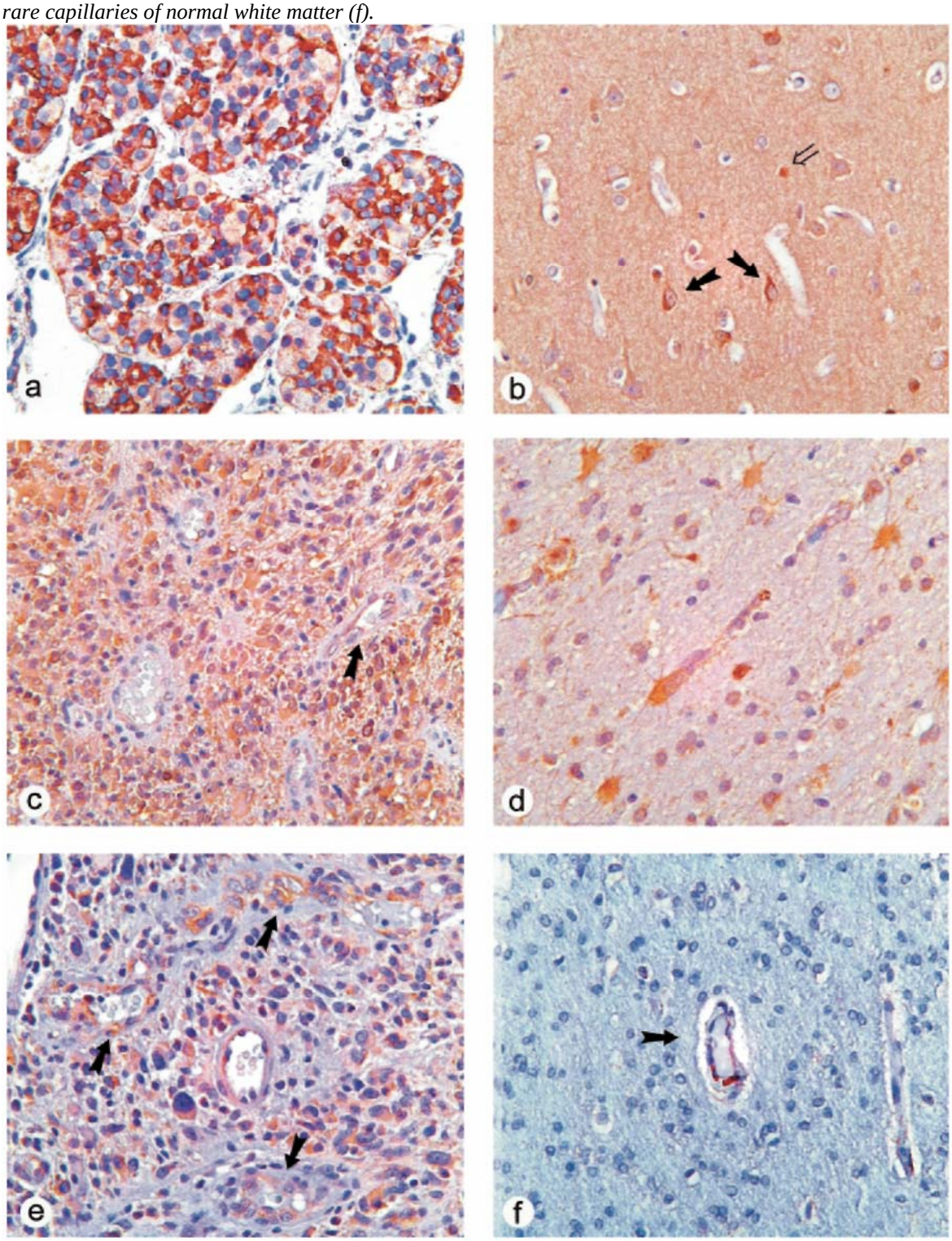

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Figure 2. *MIF mRNA quantification. (a) Representative 2% agarose gel of MIF mRNA in normal brain and GBM samples. (b) Real-time quantification for normal brain (N, n = 2) and GBMs (GB, n = 20). Values are means ± SD in each tissue sample. Experiment repeated at least three times in duplicate.*

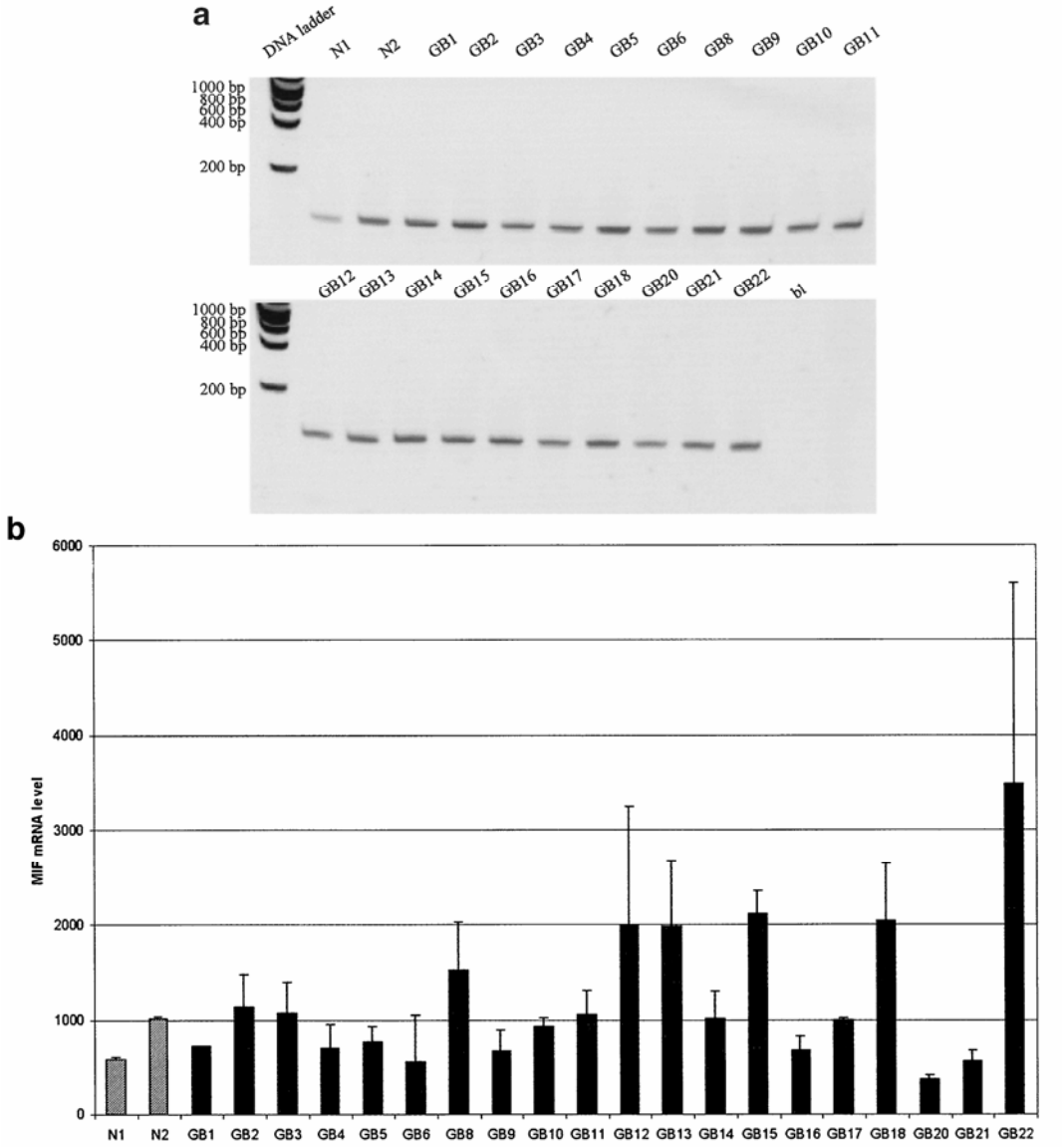

Table 2 *Immunohistochemistry for MIF and VEGF on the 22 cases also studied by RT-PCR*

	N 1	N 2	GB 1	GB 2	GB 3	GB 4	GB 5	GB 6	GB 8	GB 9	GB 10	GB 11	GB 12	GB 13	GB 14	GB 15	GB 16	GB 17	GB 18	GB 20	GB 21	GB 22
MIF	1	1	3	3	2	2	3	2	3	3	1	1	3	2	1	1	*2*	2	2	3	1	*2*
VEGF	0	1	2	2	2	1	0	2	2	2	1	2	3	2	1	1	0	2	2	3	0	*2*

N, normal brain; GB, glioblastoma. Scores for immunopositivity were read as follow: 0 = complete absence of positive cells, 1 = rare strongly positive cells, 2 = moderate density of positive cells, and 3 = strong diffuse positivity

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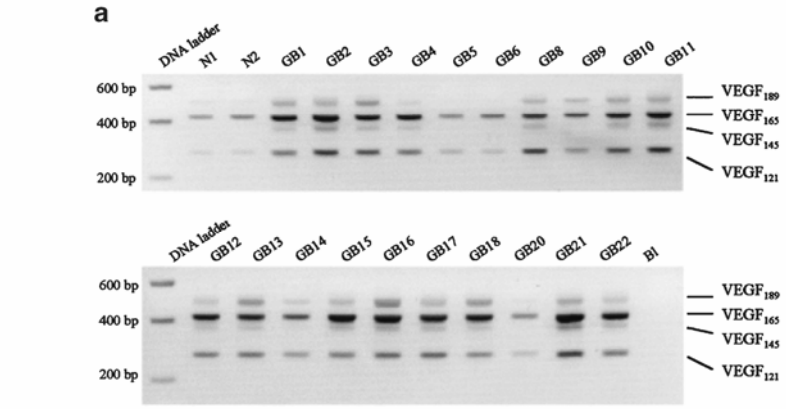
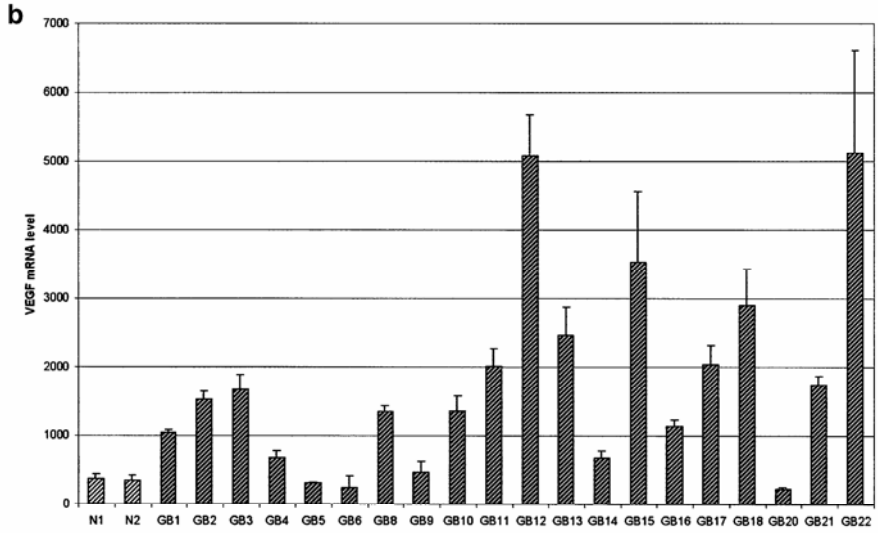
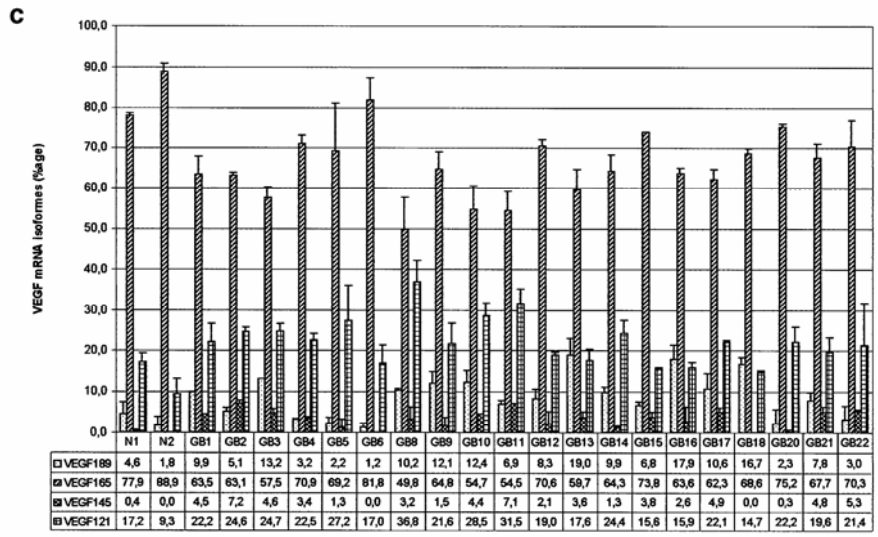
Figure 3. *VEGF mRNA quantification. (a) Representative 2% agarose gels of RT-PCR products. VEGF mRNA isoforms 189, 165, 145 and 121 are detected, respectively, at 479, 407, 347 and 275 bp. (b) Quantification of total VEGF mRNA. (c) Quantification of the isoforms of VEGF mRNA. Proportional representation (%) of each isoform. Values are means ± SD in each tissue sample. Experiment repeated at least three times in duplicate.*







	N1	N2	GB1	GB2	GB3	GB4	GB5	GB6	GB8	GB9	GB10	GB11	GB12	GB13	GB14	GB15	GB16	GB17	GB18	GB20	GB21	GB22
VEGF189	4,6	1,8	9,9	5,1	13,2	3,2	2,2	1,2	10,2	12,1	12,4	6,9	8,3	19,0	9,9	6,8	17,9	10,6	16,7	2,3	7,8	3,0
VEGF165	77,9	88,9	63,5	63,1	57,5	70,9	69,2	81,8	49,8	64,8	54,7	54,5	70,6	59,7	64,3	73,8	63,6	62,3	68,6	75,2	67,7	70,3
VEGF145	0,4	0,0	4,5	7,2	4,6	3,4	1,3	0,0	3,2	1,5	4,4	7,1	2,1	3,6	1,3	3,8	2,6	4,9	0,0	0,3	4,8	5,3
VEGF121	17,2	9,3	22,2	24,6	24,7	22,5	27,2	17,0	36,8	21,6	28,5	31,5	19,0	17,6	24,4	15,6	15,9	22,1	14,7	22,2	19,6	21,4

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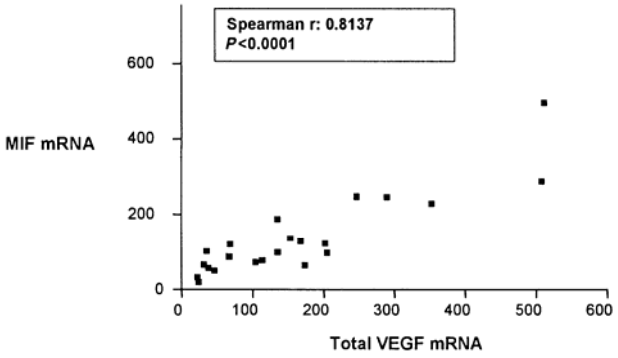
Figure 4. *Correlation between MIF and total VEGF mRNA expression. Spearman correlation coefficient.*



Discussion

In this study, we used RT-PCR and immunohistochemistry to assess the expression of MIF and VEGF in the normal human brain and in a series of 35 GBMs. We further looked for a correlation between MIF and VEGF expression. Our specimens of normal brain showed a constitutive expression of MIF. The peptide was detected mainly in neurons and in a few glial cells. MIF has been demonstrated previously in the brain of rodents in normal conditions and localized to some neurons of the cortex, hypothalamus, hippocampus, cerebellum and pons, and to a smaller component of glial cells [3,16,20,24]. MIF function in the normal brain is still largely speculative. A trophic role for MIF has been suggested, given its upregulation in the developmental stage of the rat brain [23] and its high basal expression in embryonic hippocampal and neocortical cell cultures [26]. More recently, on the basis of its enzymatic properties, MIF has been proposed as a protective factor for catecholaminergic neurons [15].

In this study, we demonstrated MIF expression in 35 GBMs, both in tumour cells and in hyperplastic endothelial cells, confirming and extending the observations of Markert *et al.* [14]. MIF expression has also been shown in other human tumours: melanoma cells [22], pituitary adenomas [25], breast cancer [4], non-small cell lung carcinoma and prostate adenocarcinoma [17]. Both *in vitro* [22] and *in vivo* [6], MIF promotes angiogenesis. Because VEGF is the most potent pro-angiogenic factor currently described in GBMs [13], we searched for a possible correlation between MIF and VEGF expression. This study indeed showed a strong correlation between MIF and VEGF, suggesting common triggering events or regulatory pathways. Inactivation of p53, a common event in the progression of gliomas [11], might be an underlying mechanism, as it has been shown to increase VEGF expression [10]. Interestingly, MIF has been reported to overcome p53-mediated cell growth arrest by inhibition of p53-dependent transcriptional activity [9]. In this study, we found no correlation between MIF expression and p53 immunoreactivity in GBMs, which is an indicator of p53 dysfunction in tumours. However, additional studies should look more carefully for a possible association, as our study included only a relatively small number of cases and did not explore p53 genetic defects. MIF could also interfere with VEGF expression through its ability to override the glucocorticosteroid inhibition of interleukin 1β (IL-1β) and IL-6, both of which promote VEGF expression *in vitro* [7,12,16]. Although the promoting effect of hypoxia on VEGF expression is still incompletely understood [8,21], we looked for a possible association between necrotic areas and MIF and/or VEGF immunostaining in the tumours. However, immunopositive cells, when present, were found throughout the tumour, with no restriction to perinecrotic areas. Finally, other tumour growth-promoting properties of MIF could also be of importance in GBMs, as MIF might also inhibit NK [2] and CTL [1] responses against tumour antigens.

In this study, peritumoral reactive astrocytes consistently showed a strong MIF labelling. High MIF expression by glial cells has been already reported in nontumoral conditions such as following experimental intracerebroventricular LPS administration [3] or in mice infected with Japanese encephalitis virus [24]. In humans, raised MIF levels were found in the cerebrospinal fluid (CSF) of patients with multiple sclerosis and neuro-Behcet's disease [18]. This is in keeping with the demonstration that physiologically, both at a systemic and a local level, MIF acts as a critical component of inflammatory and immune responses by counteracting the glucocorticoid inhibition on the acute inflammatory response [16]. In the setting of GBMs, peritumoral reactive astrocytes might represent an additional source of MIF, promoting tumour growth and resistance to glucocorticoids.

Finally, in this study, we observed no correlation between the levels of mRNA and the intensity of immunostaining for MIF. High density of MIF positive cells was found in tumours with low mRNA expression, suggesting a possible storage of MIF in tumour and/or endothelial cells. Accordingly, MIF has been shown to be stored in the secretory granules of ACTH-producing cells in the anterior pituitary [5]. In animal models,

activation of the hypothalamo-pituitary axis results in the rapid secretion of MIF from the pituitary gland into the plasma, followed by a slower time-dependent increase in the expression from pituitary MIF mRNA [5,16]. In addition, it has been reported that MIF secretion could be induced by glucocorticoids in hippocampal and neocortical rat cell cultures [26]. Therefore, the discrepancy between our results using RT-PCR and immunochemistry for MIF might reflect the effects of both cell storage and concurrent corticotherapy.

In conclusion, we showed in this study that MIF is consistently expressed in human glioblastomas to a level similar to or higher than that in the normal brain. We also observed a strong correlation between MIF and VEGF expression. MIF combines the properties of a tumour growth promoter and those of a potent counterregulator of glucocorticosteroids. Anti-MIF strategies on *in vivo* and *in vitro* models could help to determine the role of MIF as a new target in the treatment of GBMs. It would also be interesting to determine the status of MIF expression in lower grade gliomas and see how it correlates with the extent of microangiogenesis and/or histological phenotype.

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