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Adrenomedullin expression and regulation in human glioblastoma, cultured human glioblastoma cell lines and pilocytic astrocytoma

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ABSTRACT

Clinical and experimental studies suggest that angiogenesis is a prerequisite for solid tumour growth. Glioblastoma (GBM) and pilocytic astrocytoma (PA), both angiogenic tumours display strong contrast enhancement associated with peripheral oedema in GBM but not in PA indicating differences in vascular permeability in these two types of gliomas. Here we show that expression of adrenomedullin (AM) mRNA is induced in GBM whereas is barely detectable in PA. *In situ* analysis of tumour specimens undergoing neovascularisation shows that the production of AM is specifically induced in a subset of GBM cells distinguished by their immediate proximity to necrotic foci (presumably hypoxic regions), suggesting a hypoxic induction of AM expression in GBM. Vascular endothelial growth factor (*VEGF*) mRNA levels are increased in GBM and moderate in PA. Immunohistochemical study showed that cytoplasmic AM, VEGF and HIF-1α nuclear immunoreactivity were recorded in GBM located near large necrotic areas whereas they were not expressed by PA tumour cells. Interestingly, double fluorescence immunostaining demonstrated that 85% of AM immunoreactivity colocalised with VEGF. AM transduces its effects through calcitonin receptor-like receptor/receptor activity modifying protein-2 and -3 (CLR/RAMP2 and CLR/RAMP3). Real-time quantitative RT-PCR showed expression of *RAMP2*, *RAMP3* and *CLR* in PA and GBM, suggesting that AM may function as an autocrine/paracrine growth factor for GBM cells. These observations strongly support the concept that tumour angiogenesis is regulated by paracrine mechanisms and identify beside VEGF, AM as a potential tumour angiogenesis factor *in vivo* which constitutes a potential interesting molecular target in GBM treatment.

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1. Introduction

Glioblastoma multiforme (GBM, WHO grade IV), the most frequent and highly malignant primary brain tumour preferentially affects adults and due to its infiltrative behaviour it cannot be completely resected.[1] In contrast, pilocytic astrocytoma (PA, WHO grade I), the most frequent glioma in children is circumscribed and cured by total surgical excision. It occurs mainly in the cerebellum, hypothalamo-chiasmatic region and brainstem.[2] Despite highly distinctive features, both tumours are angiogenic and display strong contrast enhancement associated with peripheral oedema in GBM but not in PA indicating differences in vascular permeability in these two types of gliomas. Microvascular proliferation is a histopathological hallmark of GBM and consists of tufted aggregates of dividing endothelial cells, smooth muscle cells and pericytes.[3] However, endothelial coverage by pericytes is incomplete in GBM,[4] a feature of microvessel immaturity.[5] In addition to microvascular proliferation, foci of necrosis surrounded by poorly differentiated cells in a pseudopalisading pattern are another hallmark of GBM.[1] In contrast, microvascular proliferation rarely occurs in PA except at the edges of cyst walls but vessels are abundant and typically display hyalinised walls.

Numerous studies have reported that hypoxia is responsible for both pseudopalisading necrosis and angiogenesis in GBM.[6] Hypoxia is responsible for activation of hypoxia-inducible factor 1 (HIF-1), a transcription factor which binds to hypoxia-responsive elements (HRE) of various target genes in reduced oxygen condition.[7–9] In GBM, the heterodimer HIF-1α/HIF-1β is responsible for the up-regulation of vascular endothelial growth factor (VEGF) and it has been reported to up-regulate adrenomedullin (AM) gene expression under hypoxia in T98G cells.[10] Hypoxic induction of VEGF is considered to be the major driving force behind new vessel development both during embryogenesis and tumour angiogenesis and the VEGF signalling pathway is the major target of currently available anti-angiogenic therapies.[11]

AM is a multifunctional peptide with properties ranging from inducing vasorelaxation to acting as a regulator of cellular growth.[12] AM transduces its effects through the G protein-coupled receptor calcitonin receptor-like receptor (CLR), with specificity for AM being conferred by the receptor activity modifying protein-2 (RAMP2) and -3 (RAMP3).[13] The ability of CLR/RAMP2 and CLR/RAMP3 to respond with high affinity to AM implies the existence of two molecularly distinct AM receptors referred to as AM_1 and AM_2 receptors, respectively.[14] AM is expressed in a variety of malignant tissues and was shown to be mitogenic for human cancer cell lines including lung, breast, colon, glioblastoma and prostate lineages *in vitro*.[15,16]

The aim of this study was to analyse the expression of AM and its receptors CLR, RAMP2 and RAMP3 in a large cohort of GB and PA in order to evaluate the molecular differences between the vasculature of these two angiogenic gliomas. In addition, we analysed AM topographic distribution using *in situ* hybridisation and immunohistochemistry in comparison to those of VEGF and HIF-1α. Finally, we assessed in two glioblastoma cell lines (U87, U373) and in primary glial cell cultures, the effects of hypoxia on AM expression.

2. Materials and methods

2.1. Human tumour samples

All patients were operated at the same institution (Assistance Publique-Hôpitaux de Marseille, Marseille, France) and written informed consent was obtained in each case. All tissue procurement protocols were approved by the relevant institutional committees. Molecular analysis using real-time quantitative-RT-PCR (Q-RT-PCR) was performed on 69 frozen gliomas classified according to the WHO CNS tumour classification[1] as glioblastomas (GBM, grade IV, $n = 52$) and pilocytic astrocytomas (PA, grade I, $n = 17$). In addition, five GBM samples and three PA samples were fixed in 4% paraformaldehyde, cryoprotected in 20% sucrose, then frozen in melting isopentane and stored at −80 °C for *in situ* hybridisation analysis. The same samples were formalin-fixed and paraffin-embedded for immunohistochemistry and immunofluorescence studies.

2.2. RNA preparation and real-time quantitative RT-PCR (Q-RT-PCR)

Histological control on cryostat section was always realised before tumour pulverisation. All PAs contain 100% of tumoural tissue whereas GBM contains at least 60% of tumoural tissue and less than 40% of necrosis. Total RNA was extracted with a phenol–chloroform method[17] as previously described.[18] Forward and reverse primers and probes to quantify genes with their corresponding PCR conditions are described in Table 1.

2.3. AM in situ hybridisation

In situ hybridisation using 35S-labelled riboprobes was performed as described previously.[19] Observation was done in a Leitz DMRD (Wetzlar, Germany) light microscope equipped with a planachromatic 20×/0.50 objective. Representative microscopic fields were captured as indicated for immunocytochemistry, except that each field was captured twice: once under bright field microscopy to assess histopathological characteristics and once under dark-field microscopy for detecting the distribution of the radioautography silver grains.

2.4. Immunohistochemistry of HIF-1α, AM and VEGF

We examined the expression of HIF-1α, AM and VEGF proteins in GBM patients ($n = 5$) who present high expression of AM and VEGF by Q-RT-PCR analysis. PAs were taken as negative control ($n = 3$). Immunohistochemistry was carried out on formalin-fixed paraffin-embedded samples. Five micrometre sections were tested after heat-induced antigen retrieval (97 °C, 40 min, citrate pH 6 buffer). Anti-AM antibody was used at 1/750 dilution[16] and incubated overnight at 4 °C before using an avidin-biotin complex kit (Histostain plus, Zymed). Endogen peroxidase was neutralised by 3% H_2O_2. Anti-HIF-1α polyclonal antibody (1/500, gift from J. Pouyssegur, Nice, France)[20] and anti-VEGF antibody (1/100; RD systems, France) were used for immunostaining performed using a Ventana Automate (Ventana Medical Systems SA, Illkirch, France).

Table 1 – Sequences of forward and reverse primers and probes used to quantify human *AM*, *VEGF*, *CLR*, *RAMP2*, *RAMP3* mRNAs, and *18S* rRNAs. Corresponding Q-RT-PCR conditions are also given.

Gene	Primers, fluorescent probes, PCR conditions
AM	Forward primer 5′-TGCCCAGACCCTTATTCGG-3′ Reverse primer 5′-AGTTGTTCATGCTCTGGCGG-3′ Probe FAM-ACATGAAGGGTGCCTCTCGAAGCCC-TAMRA (95 °C for 15′; 45 cycles of 94 °C for 15″, 67 °C for 20′′)
VEGF	Forward primer 5′-AGGAGGAGGGCAGAATCATCA-3′ Reverse primer 5′-AGGGTCTCGATTGGATGGC-3′ Probe FAM-TGAAGTTCATGGATGTCTATCAGCGCAGCT-TAMRA (95 °C for 15′; 45 cycles of 94 °C for 15″, 66 °C for 15′′)
CLR	Forward primer 5′-TGGCTTAATGATGGAGAAAAAGTG-3′ Reverse primer 5′-TCAGGACTCTCTTCTAATTCTGCTG-3′ Probe FAM-CCTGTATTTTCTGGTTCTCTTGCCTTTTTTTTTATGA-TAMRA (95 °C for 15′; 40 cycles of 94 °C for 20″, 60 °C for 20′′)
RAMP2	Forward primer 5′-GACGGTGAAGAACTATGAGACAGC-3′ Reverse primer 5′-GCTATAAGGCCTGCTAATCATGG-3′ Probe FAM-TGGATCCTATCGAAAAGGATTGGTGCG-TAMRA (95 °C for 15′; 40 cycles of 94 °C for 10″, 65 °C for 15′′)
RAMP3	Forward primer 5′-TCTGGAAGTGGTGCAACCTGT-3′ Reverse primer 5′-GATGCCGGTGATGAAGCC-3′ Probe FAM-AGATGGAGGCCAATGTCGTGGGCT-TAMRA (95 °C for 15′; 40 cycles of 94 °C for 20″, 67 °C for 30′′)
18S	Forward primer 5′-CTACCACATCCAAGGAAGGCA-3′ Reverse primer 5′-TTTTTCGTCACTACCTCCCCG-3′ Probe FAM-CGCGCAAATTACCCACTCCCGAC-TAMRA (95 °C for 15′; 40 cycles of 94 °C for 15″, 67 °C for 15′′)

FAM, 5-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine.

2.5. *Double immunofluorescence staining of AM and VEGF in GBM*

Ten micrometre sections of GBM samples were incubated in 5% albumin from bovine serum/PBS (Sigma–Aldrich) for 30 min. They were then incubated with the primary antibody mixture (AM diluted at 1/1000; VEGF diluted at 1/100) in 2% bovine serum albumin and 0.1% Triton X-100 in PBS for 1 h at room temperature and the secondary antibody anti-goat IgG FITC conjugate and the anti-rabbit Texas Red conjugate (Jackson) diluted at 1/100. Sections were visualised under a Leica (DLMB) immunofluorescence microscope. The staining was semi-quantitatively assessed to determine the percentage of labelled cells.

2.6. *Western blot analysis*

Aliquots of protein extracts (40 μg) of GBM tissues ($n = 2$) were separated on 12% of SDS-PAGE, transferred to H–C membrane and immunoblotted using antibodies generated against CLR, RAMP2 and RAMP3 peptides as previously described.[21]

2.7. *Cell culture and hypoxia study*

Human glioblastoma cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in minimum essential medium (U373 and U87). Cells were cultured at 37 °C under moist 5% CO_2/95% air atmosphere, and fed with fresh medium every 2 days, being routinely monitored for mycoplasma contamination (Roche Molecular Biochemicals, Meylan, France). All experiments were begun at about 70% confluency. To examine the effect of hypoxia on the levels of AM mRNA and immunoreactive AM (ir-AM), U373 and U87 cells were placed in a chamber filled with 5% CO_2/94% N2/1% O_2 at 37 °C. The cells were cultivated for 6 to 24 h and were harvested for RNA extraction. The culture medium was also collected for the measurement of ir-AM. In another series of experiments, cells were exposed to 150 μmol/L of cobalt chloride ($CoCl_2$) or 260 μmol/L of desferrioxamine mesylate (DFX) for 6 to 24 h and then harvested for RNA extraction. All culture media components were purchased from Invitrogen Life Technologies (Paris, France).

2.8. *Peptide extraction and radioimmunoassay*

Medium from primary tumoural glial cells and U87 cell line was prepared for radioimmunoassay (RIA) of AM as previously described.[16] The RIA of AM was performed as reported previously,[22] using the antiserum against human (AM 1–52) amide developed in our laboratory and used at a final dilution of 1:30,000. To measure the ir-AM in the culture medium, the medium was extracted by the previously reported method[22] using Sep-PaK C18 cartridges (Waters, Milford, MA).

2.9. *Statistical analysis*

Statistical analyses were conducted using the statistical Package SPSS software v.15 (SPSS Inc., Chicago, USA). Quantitative

RT-PCR data were expressed as mean ± the standard error of the mean (SEM), and differences between GBM and normal telencephalon were analysed using the Mann–Whitney U test. The difference was considered significant if the *p*-values were less than 0.05.

3. Results

3.1. *Increased expression of AM and VEGF mRNAs in glioblastomas (GBMs) in comparison to pilocytic astrocytomas (PAs)*

Quantification of *AM* mRNA transcripts on large number of samples revealed high *AM* mRNA levels in GBM as compared to PA, in agreement with our previous data on small number of GBM.[16] The *AM* mRNA expression analysis demonstrated a significant difference between GBM and PA (58.2 ± 8 fg/ng 18S rRNA in GBM versus 5.9 ± 1.7 fg/ng 18S rRNA in PA ($p < 0.0001$)) (Fig. 1A). The mean level of *VEGF* mRNA was increased in GBM (162.3 ± 19 fg/ng 18S rRNA) as compared to PA (19.9 ± 5.7 fg/ng 18S rRNA, $p < 0.0001$) (Fig. 1A). *VEGF* mRNA expression was correlated with *AM* mRNA expression in PA and GBM, (respectively, 0.75, $p < 0.001$; 0.56, $p < 0.0001$, Pearson Correlation Test).

To determine whether AM was predominantly expressed in a restricted subpopulation of tumour cells, *in situ* analysis of *AM* mRNA was performed to identify the producer cells. In Fig. 1B, *AM* mRNA is seen to be mainly produced in a fraction of tumour cells (presumably experiencing the most severe hypoxia) arranged in a stripe-like pattern alongside the periphery of necrotic regions. The same finding has been previously reported for VEGF expression in GBM.[23]

3.2. *Distribution of HIF-1α, AM and VEGF immunostaining in GBM*

HIF-1α, AM and VEGF proteins expressions were recorded in tumour cells in all samples studied although the proportion of cells expressing HIF1-α, AM and VEGF varied considerably between tumours. As expected, HIF1-α nuclear immunoreac-

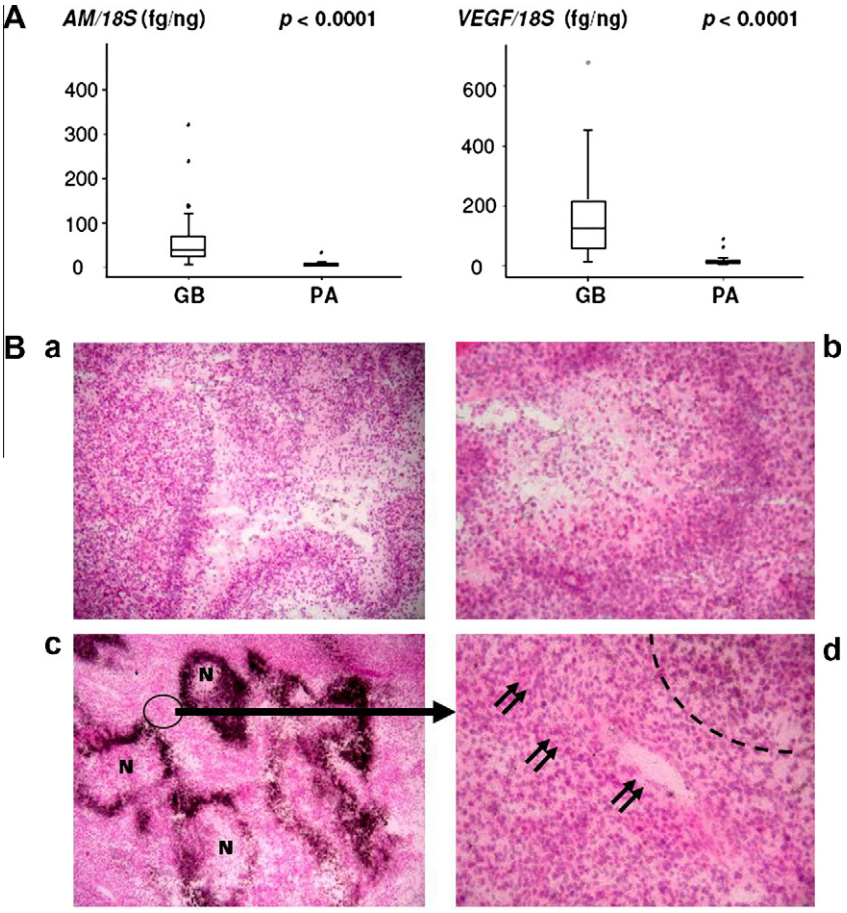

Fig. 1 – Expression of AM and VEGF in GBM and PA. (A) Real time quantitative RT-PCR analysis of *AM* and *VEGF* mRNA levels in GBM and PA. Total DNA-free RNAs from the GBM ($n = 52$) and PA ($n = 17$) were transcribed to cDNA and subjected to quantitative RT-PCR using the ABI Prism 7700 sequence detection system for the estimation of relative *AM* and *VEGF* mRNA to 18S rRNA ratio as described in Section 2. (B) Radioactive *in situ* hybridisation of AM mRNA in a human GBM. A thin section of GBM tumour hybridised with the sense AM riboprobe showing no signal (high specificity) at magnification 20× (a) et 40× (b) and with the antisense AM riboprobe at magnification 20× (c) showing a strong signal. AM expressing cells are localised alongside the edges of necrotic regions (N) and a high endothelial proliferation can be observed at the periphery of these zones (d). Furthermore, the clusters of silver grains concentrate within tumour cells rather than in endothelial cells (d).

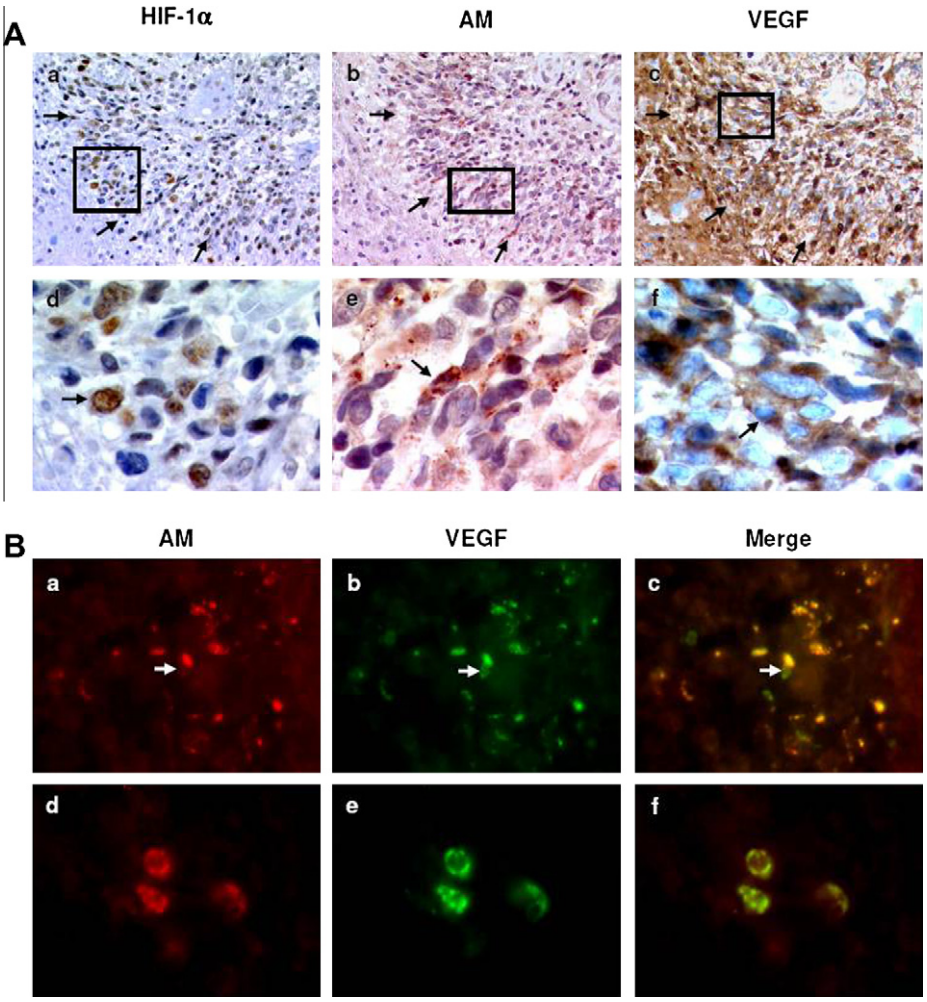

Fig. 2 – HIF-1α, AM and VEGF proteins are present in GBM *in vivo*. (A) Immunocytochemical staining for HIF-1α (a), AM (b) and VEGF (c) shows strong immunoreactivity in palissading cells that reside along necrosis. (d–f) Enlargement of the areas boxed in a–c, respectively. (B) Immunofluorescence of AM and VEGF demonstrates that in GBM, 85% of AM immunoreactivity colocalise with VEGF.

tivity was expressed frequently in GBM cells located near large necrotic areas and in some pseudopalisading tumour cells (Fig. 2A a and d) as well as cytoplasmic AM (Fig. 2A b and e) and VEGF (Fig. 2A c and f). Interestingly, double fluorescence immunostaining demonstrated clearly that in GBM, 85% of AM immunoreactivity colocalised with VEGF (Fig. 2B) whereas 6% and 9% of cells exclusively expressed AM or VEGF, respectively. Moreover, immunostaining of HIF-1α, AM and VEGF was not recorded in PA tumour cells (data not shown).

3.3. *RAMPs and CLR are expressed in GBM and PA*

Fig. 3A shows that *CLR*, *RAMP2* and *RAMP3* mRNAs were expressed in all of the samples prepared from GBM and PA. No significant difference in the expression of *CLR*, *RAMP2* and *RAMP3* mRNAs could be observed between GBM and PA. Omission of the reverse transcriptase eliminated the signal, which indicated that it was not attributable to contaminating genomic DNA (data not shown).

To confirm the expression of CLR, RAMP2 and RAMP3 proteins, protein extracts (40 μg) from tumour cells of two GBM samples were subjected to western blot analysis (Fig. 3B). The specificity of our immunodetection assay was confirmed by an antibody adsorption control that eliminated the specific bands (data not shown).

Taken together, these findings demonstrate that CLR, RAMP2, and RAMP3 mRNAs and proteins are expressed in GBM tumoural cells and may contribute to the function of AM.

3.4. *Regulation of AM expression in human glioblastoma cells by hypoxia*

The two cell lines (U373, U87) exposed to hypoxia, $CoCl_2$, and DFX demonstrated a consistent induction of AM expression, and Fig. 4 illustrates a representative example of the observed response in our tests conditions (hypoxia, exposure to 150 μmol/L $CoCl_2$ and to 260 μmol/L DFX).

Of the three treatments, exposure to 1% O_2 and to DFX showed a steeper induction of AM mRNA over time, and also more dramatic increases between the basal and the maximum induction were observed (>28- and 25-fold increase

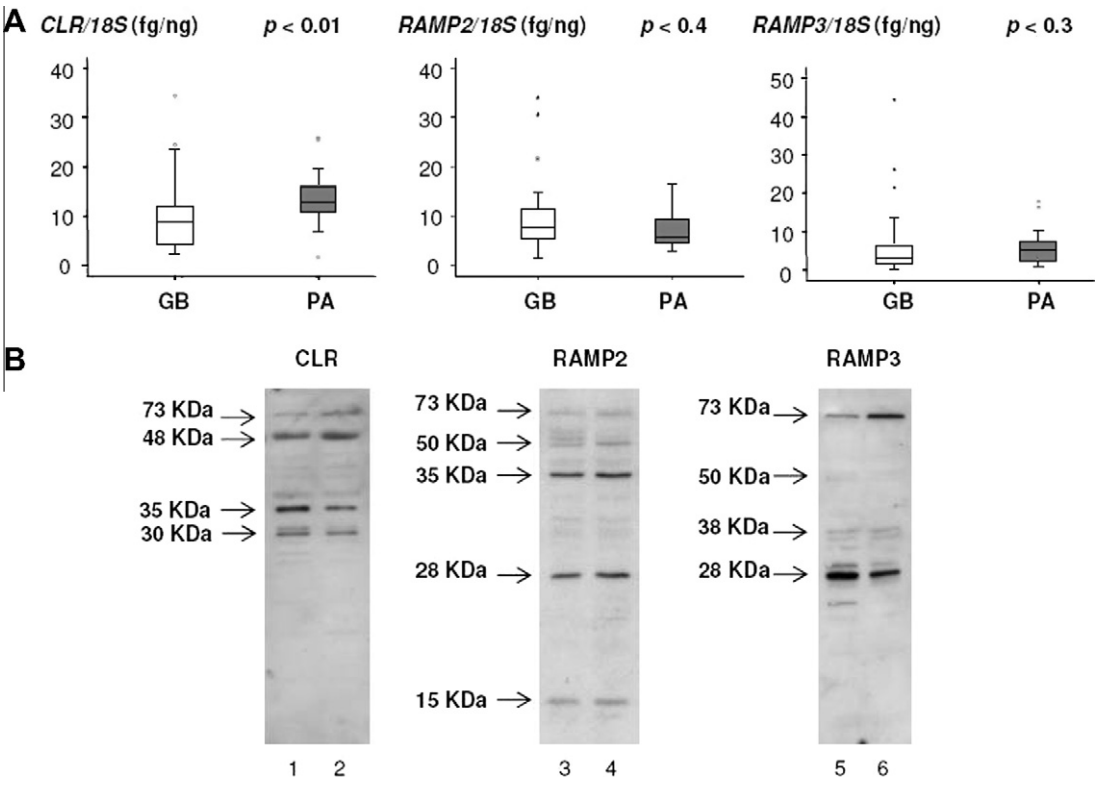

Fig. 3 – Expression of CLR, RAMP2 and RAMP3 in GBM and PA. (A) Real-time quantitative RT-PCR analysis of CLR, RAMP2 and RAMP3 mRNA levels an GBM (n = 52) and PA (n = 17). Total RNA DNA-free from GBM and PA was reverse transcribed and quantified as described in for the estimation of relative *CLR*, *RAMP2* and *RAMP3* mRNAs to 18S rRNA ratio as described in Section 2. In GBM, the mean level of CLR, RAMP2 and RAMP3 mRNAs expression was 9.9 ± 0.9 fg/ng 18S rRNA, 9.3 ± 0.9 fg/ng 18S rRNA, and 5.6 ± 1.1 fg/ng 18S rRNA, respectively. In PA, the mean level of *CLR*, *RAMP2* and *RAMP3* mRNAs expression was 13.9 ± 1.6 fg/ng 18S rRNA, 7.7 ± 1.1 fg/ng 18S rRNA, and 6.2 ± 1.3 fg/ng 18S rRNA, respectively. No significant difference in the expression of *CLR*, *RAMP2* and *RAMP3* mRNAs could be observed between GBM and PA. (B) Aliquots of protein extracts (40 μg) of GBM tissues (n = 2) were separated on 12% of SDS-PAGE, transferred to H–C membrane and immunoblotted using antibodies generated against CLR, RAMP2 and RAMP3 peptides as described. GBM produced CLR as a distinct band of 48 kDa after SDS-PAGE and immunoblotting (B, lanes 1 and 2). RAMP2 ran as a monomer of 15 kDa and multimer presumably homodimer at 50 kDa (B, lanes 3 and 4). The 35 kDa band may represent a heterogeneously glycosylated form of RAMP2 (B, lanes 3 and 4). RAMP3 ran as an heterogeneously glycosylated monomer of 28 kDa and an heterodimer of 73-kDa (B, lanes 5 and 6). Interestingly, the 73 kDa band can be revealed by the three antibodies and must correspond to heterodimers CLR/RAMP2 and CLR/RAMP3 (B).

between maximum induction and baseline levels for exposure to 1% O_2 and DFX, respectively).

We also determined the ir-AM in the conditioned medium of U373 and U87 cells and primary glial cell culture prepared from 8 GBM specimens under hypoxic condition. ir-AM accumulated in the culture media of U373 and U87 cells and primary glial cell culture time-dependently up to 24h under normoxia and hypoxia. The ir-AM levels were significantly higher under hypoxia than under normoxia in primary glial cell culture (Fig. 5), U87 (151.6 ± 22.4 *versus* 47.9 ± 12.1 fmol/10^6 cells) and U373 (169.5 ± 27.3 *versus* 54.7 ± 9.6 fmol/10^6 cells) cells (not shown). Similarly results were obtained with the conditioned media of these cell lines for $CoCl_2$ or DFX treatments (not shown).

4. Discussion

In this paper, we reported a strong AM expression in GBM comparing to PA ($p < 0.001$). In contrast, RAMP2 and RAMP3 expressions were in the same range in both tumours whereas CLR expression was higher in PA than in GBM ($p < 0.01$). High AM expression in GBM in contrast to grade II and III infiltrative glioma was previously reported by our group, but at this time we did not study AM expression in PA.[16] The marked differences in steady-state levels of *AM* mRNA amongst otherwise indistinguishable tumour cells (Fig. 1B) can be correlated with their proximity to necrotic centres, where oxygen supply is minimal. This observation can be interpreted to mean that AM could be specifically induced in response to hypoxia. Interestingly, Garayoa and coworkers[24] demonstrated that the expression of *AM* mRNA in a variety of human cell lines is highly induced by hypoxia. Here, we also demonstrated that reduced oxygen tension (1% O_2) or exposure to hypoxia mimetics such as desferrioxamine mesylate (DFX) or $CoCl_2$, induced *AM* mRNA expression in U87 and U373 GBM cell lines. The increase in *AM* mRNA levels was reversible. Upon re-exposure of cells to normal oxygen

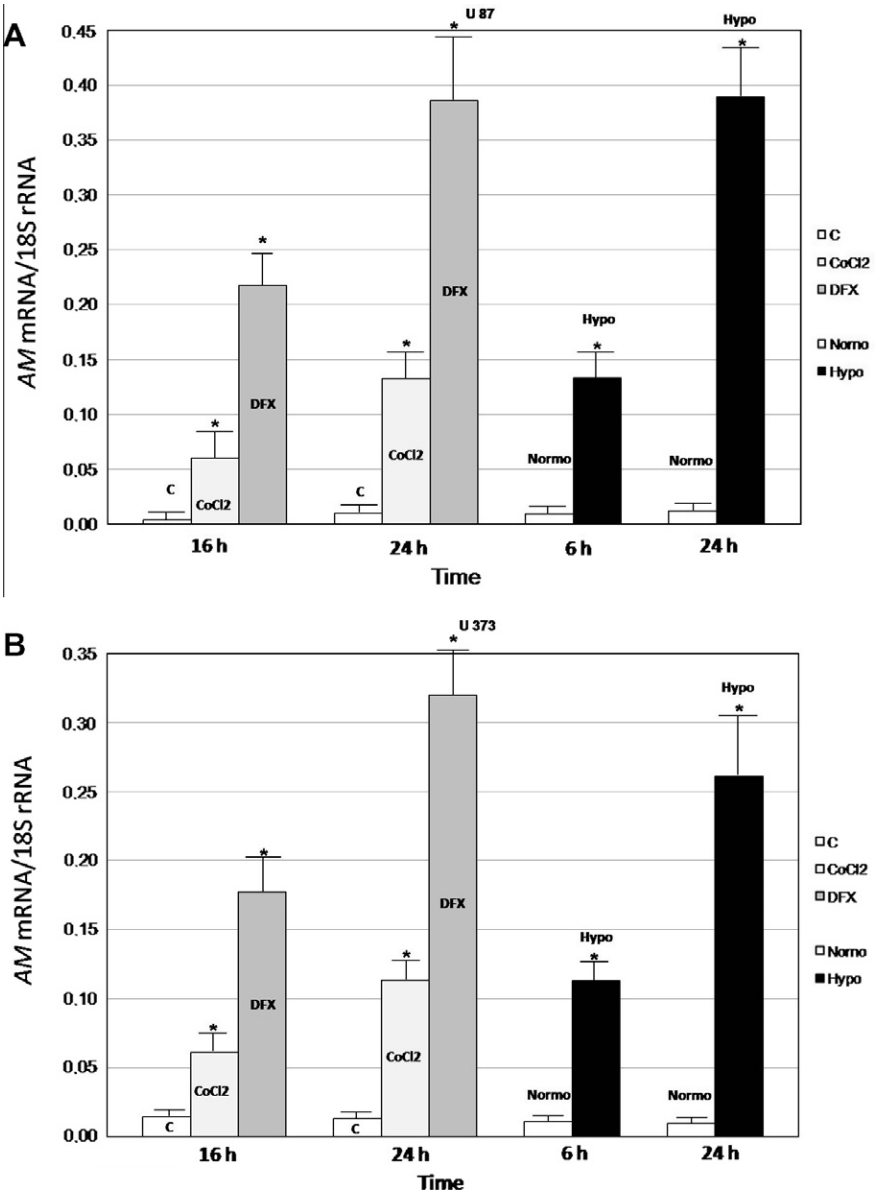

Fig. 4 – (A): Hypoxic induction of AM mRNA in U87 (A) and U373 (B) glioblastoma cells. The intensity of hybridisation signals of northern blot analysis was quantified with a Bioimage analyser, and the intensity of representing AM mRNA was normalised with respect to the intensity for 18S r-RNA. The ratio of each normalised value to the control value (before hypoxic experiments) is shown as the relative expression level of AM mRNA. The data are means ± SEM. The time course study revealed that expression levels of AM mRNA increased significantly after 6-h exposure to hypoxia and reached the maximum at the 24-h time point (about 32- and 28-fold increase compared to control for U87 and U373, respectively). In the hypoxia mimetics experiment ($CoCl_2$, DFX), the difference of AM mRNA expression was significant at 16-h time point and reached the maximum at the 24 h time point. The mean calculated test/basal ratio was of 14- and 9-fold in $CoCl_2$ exposure experiments for U87 and U373 cell lines, respectively. It was of 37- and 25-fold DFX exposure experiments for U87 and U373 cell lines, respectively.

tension, AM expression resumed its low constitutive level (not shown). These findings support the thesis that the induction of AM in GBM occurs in response to hypoxia. They also indicate that the rate of release of angiogenic factors (AM, VEGF…) by tumour cells might in general be variable, being constantly adjusted according to the changes in the cell microenvironment.

Evidence for both AM and AM receptors in GBM favours that AM functions as autocrine/paracrine growth factor in GBM. In agreement, anti-AM antibody significantly decreased *in vitro* and *in vivo* growth of U87 glioblastoma cells.[16] In addition, to its role in tumour progression, AM knockout mice suggest that AM is essential for vascular morphogenesis[25] and also tumour angiogenesis.[26] In various tumour models, AM expression correlates with vascular density[16,27] but highly vascularised tumour may express low level of AM as reported here for PA. In fact, the mechanisms leading to angiogenesis in both GBM and PA are strikingly different: in PA, which is a

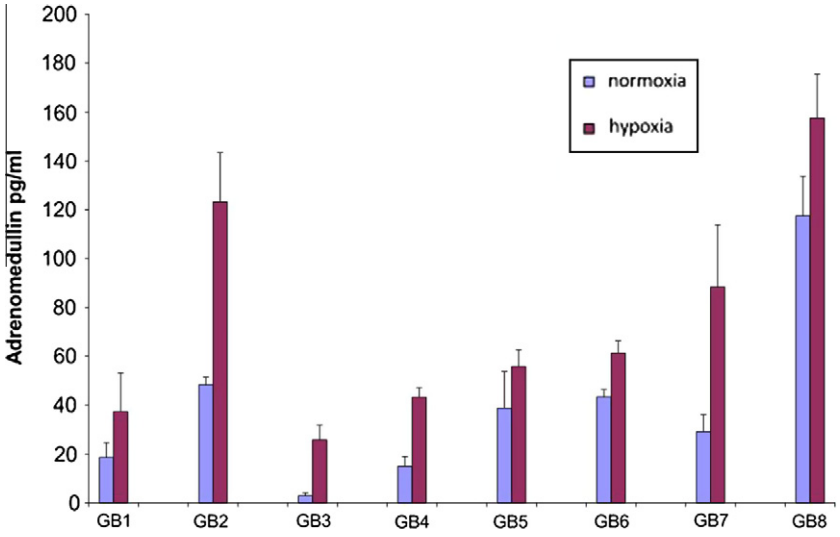

Fig. 5 – Effect of hypoxia on AM protein level in primary glial cell culture prepared from 8 human glioblastoma specimens. IR-AM levels in the culture medium under normoxic and hypoxic conditions. The data shown are means ± SEM. $p < 0.005$.

slowly growing tumour, preexisting blood vessels are sufficient to ensure tumour growth. Vessels diameter increased and become abnormal with hyalinised walls as tumours enlarged. In contrast, in rapidly growing GBM, blood supply is not sufficient leading to necrosis and hypoxia, which is the major trigger of angiogenesis. Increased AM expression in hypoxic condition occurs through increased transcription of AM gene triggered by HIF-1 (HRE binding in the promoter region of AM gene) and also through increase of AM mRNA stability that takes place during hypoxia.[28] Under hypoxic conditions, VEGF is also upregulated through HIF-1 binding.[9] In addition, AM administration upregulates the expression of VEGF in both *in vivo* and *in vitro* models.[26] In contrast, blocking antibodies to VEGF cannot significantly inhibit AM induced capillary tube formation by HUVEC indicating that AM expression is to some extent independent to VEGF.[29] In our study AM and VEGF mRNA expressions were highly correlated ($p < 0.001$) but strikingly different from one GBM to another. Moreover, immunohistochemistry shows that the expression pattern of these markers varies from one GBM to another. In some cases it was diffuse but in others, restricted to some areas exhibiting palisading necrosis. In these areas, the pattern of VEGF and AM expression observed in GBM only partly overlap with that of HIF-1α; HIF-1α expression was restricted to some pseudopalisading tumour cells adjacent to necrosis whereas AM and especially VEGF expression was more widespread.

Tumour vessels in GBM are structurally and functionally abnormal. They are tortuous with leaking architecture and irregular diameter and walls and contribute to the pathogenesis of tumour-associated oedema. A relative deficiency of pericytes or pericytes function could be responsible for this morphological feature.[4] The number of α-smooth muscle actin expressing cells, a marker of pericytes, was much lower in anaplastic astrocytomas than in PA which is made of large, regularly shaped and still mature vessels.[30] However, there are increasing evidences that remaining functional vessels in GBM are at least in part responsible for resistance to anti-angiogenic therapy since inhibition of VEGF signalling can lead to substantial reduction in tumour vascularity but does not affect vessels highly covered with pericytes.[31] Therefore, there is a rationale for targeting both endothelial cells and pericytes.[32] In contrast to VEGF induced angiogenesis which is not associated with vessel maturation, AM induced angiogenesis facilitates formation of mature vessels that include vascular smooth muscle cells.[33] Therefore, there is a rationale for angiogenesis inhibitors targeting the AM signalling pathway in GBM. Moreover, it is possible that AM in GBM contributes to tumour associated oedema because of its strong vasodilator action.[15] In addition to AM, it has been shown that hypoxia also upregulated CLR expression in microvascular endothelial cells although RAMP expression is not affected by hypoxia in microvascular cells.[34] In agreement, RAMP2 and RAMP3 expressions did not differ in PA and GBM. Surprisingly, however, CLR expression was higher in PA in comparison to GBM suggesting that another factor different from hypoxia may govern CLR expression in PA.

As a conclusion, in PA which is a circumscribed and slow growing tumour, preexisting vessels adapt their calibre and vascular pressure to ensure sufficient blood supply whereas in highly infiltrative and rapidly growing GBM, hypoxia-dependent angiogenesis occurred involving numerous growth factors including AM and its receptors. Targeting AM signalling might be instrumental for anti-angiogenic therapy in these tumours.

Conflict of interest statement

None declared.

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