Differential downregulation of vascular endothelial growth factor by dexamethasone in normoxic and hypoxic rat glioma cells

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Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is a mitogen and chemotactic factor for endothelial cells *in vitro* and an angiogenesis and vascular permeability factor *in vivo*. Due to its properties, VEGF is a candidate for both angiogenesis and vascular permeability/oedema induction which typically occur in glioblastomas. In this study we test the hypothesis that the antioedema effect of dexamethasone is mediated by downregulation of VEGF or VEGF receptor expression. VEGF mRNA and protein levels of two rat glioma cells lines, C6 and GS-9L, were determined after incubation with dexamethasone under normoxic and hypoxic conditions. In normoxic C6 and GS9L cells, we observed 50–60% downregulation of VEGF mRNA by dexamethasone ($P=0.015$ and $P=0.01$, respectively). This effect was dependent on glucocorticoid-receptor (GR) function. The inhibitory effect of dexamethasone on VEGF gene expression by tumour cells was markedly reduced by hypoxia which suggests that the upregulation of VEGF driven by hypoxia overcomes the effect of the dexamethasone. Dexamethasone did not alter VEGFR-2 mRNA levels in human umbilical endothelial cells. In a subcutaneous glioma tumour model, we observed only a 15% decrease in VEGF mRNA expression in dexamethasone treated animals ($n=12$) compared with controls animals ($P=0.24$). We conclude that dexamethasone may decrease brain tumour-associated oedema by reduction of VEGF expression in tumour cells. However, the highly reduced activity on hypoxic tumour cells suggests that dexamethasone efficacy may be limited by hypoxia in rapidly growing tumours.

Keywords: VEGF, dexamethasone, brain tumour, brain oedema, gliomas

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Introduction

A common feature of malignant brain tumours is their ability to disturb the blood–brain barrier (BBB) and to increase capillary permeability which subsequently leads to vasogenic brain oedema. Vasogenic brain oedema contributes substantially to the increase in brain volume leading to elevated intracranial pressure. Since their introduction as antioedema drugs more than 30 years ago, glucocorticoids have served as major therapeutic substances for peritumoural brain oedema [12]. For this reason, a large amount of research has been directed toward the elucidation of the possible mechanism by which glucocorticoids mediate their antioedema effects. It is generally accepted that the favourable effects of steroids are primarily related to a reduction in the permeability of a disrupted BBB [22]. Possible mechanisms that have been proposed to account for such an effect include inhibition of phospholipase A2,

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stabilization of membrane lysosomes, and improvement in peritumoural microcirculation [39]. Although many efforts to clarify this issue have been undertaken, the mechanism of steroid action on tumour-associated oedema is still not well defined.

It is also uncertain by which mechanism brain tumours disrupt the BBB and induce vascular permeability [6]. Several studies show that the increased vascular permeability in brain tumours is associated with morphological alterations in tumour capillaries. These morphological alterations include the occurrence of fenestrae, defective tight junctions, increased numbers of pinocytotic vesicles and incomplete ensheathment of endothelial cells by the basal membrane [15,24]. The 36–46 kDa dimeric glycoprotein, vascular endothelial growth factor, has gained attention as a possible key factor in inducing vascular permeability [6,13]. VEGF is a specific mitogen for endothelial cells and has been involved in numerous physiological and pathological settings like embryonic angiogenesis [17], corpus luteum angiogenesis [19], proliferative retinopathy [1] and tumour angiogenesis [20]. Beside its mitogenic activity, VEGF possesses a unique and strong vascular permeability activity in the Miles-Assay, being about 1000 times more potent than histamine [30]. VEGF is therefore also denominated vascular permeability factor (VPF). Roberts and Palade have provided direct evidence that VEGF induces morphological changes in endothelial cells which are consistent with an increase in vascular permeability, such as an increase in fenestrae and pinocytic vesicles [28].

Several reports suggest a correlation between VEGF expression and oedema formation in a variety of brain tumours. *In vivo*, VEGF is upregulated in highly vascularized, oedema-associated tumours like malignant gliomas and metastases [2,26,34]. Moreover, VEGF expression has been found to be increased in tumour-associated cysts suggesting that it plays a role in the genesis of vascular permeability associated with tumour growth [33,37]. We have recently observed that VEGF expression is correlated with vascular permeability in human gliomas *in situ* (M. Machein *et al.*, unpublished observations).

Recent findings in the rat female reproductive system suggest that VEGF mRNA expression is hormonally regulated in steroid-producing and steroid-responsive cells [7,31].

Based on these data, we investigated the hypothesis that dexamethasone, the most commonly used steroid for the treatment of tumour-associated oedema, mediates its effect by downregulating the expression of VEGF or VEGF receptors.

Materials and methods

Cell lines and culture conditions

C6 cells were obtained from the American Type Culture Collection. GS9L cells were a gift from Tom Budd, St Lawrence University. C6 cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. GS9L cells were cultured with RPMI1640 medium supplemented with 10% FCS and penicillin/streptomycin. Human umbilical vein endothelial cells (HUVEC) were isolated as described [5], plated in 2% gelatine-coated plates and cultured in M199-Medium supplemented with 10% FCS.

Twenty-four hours prior to *in vitro* experiments FCS was reduced to 1%. Cells were then incubated either overnight (hydrocortisone, oestradiol, progesterone) or for various time points (dexamethasone) with the following concentrations of the test substances: 1 nmol/l, 10 nmol/l, 100 nmol/l, 1 µmol/l, 5 µmol/l and 10 µmol/l dexamethasone (Merck, Darmstadt); 10 µmol/l hydrocortisone; 10 µmol/l oestradiol and 10 µmol/l progesterone (Sigma, Deisenhofen). To inhibit dexamethasone effects, the glucocorticoid specific antagonist RU 43044 (gift of Roussel UCLAF, Paris, France) was coincubated with 10 µmol/l dexamethasone at an equimolar ratio.

Expression of glucocorticoid receptor in C6 and GS9L glioma cells *in vitro* and *in vivo*

To determine whether rat glioma cells express glucocorticoid receptors, indirect immunofluorescence was performed using the monoclonal antiglucocorticoid receptor antibody GR 49/4 [38]. Cells were grown on 35 mm glass cover slips placed in 100 mm tissue culture plates. On subconfluence, cells were treated with dexamethasone 1 µmol/l overnight. Subsequently, cells were washed in phosphate buffer (PBS), fixed in methanol at −20 °C for 4 min and incubated for 1 h with the primary antibody. After washing in PBS, cells were incubated with a goat-anti mouse Cy3 secondary antibody. Microphotographs were taken with a Zeiss Axiophot.

A similar experiment was carried out with subcutaneously transplanted C6 and GS9L gliomas. For this experiment, 10^7 glioma cells were injected subcutaneously into Sprague-Dawley (for C6) or Fisher rats (for GS9L). Fourteen days later, animals were killed, the tumour was removed and snap-frozen. Acetone-fixed cryosections (10 μm) were stained as described above.

Hypoxia assay

For the hypoxia experiments, C6 and GS9L cells were cultured in an anaerobic (oxygen content approximately 0.2%) gas chamber (Becton Dickinson) as previously described [25]. Incubations were carried out overnight (16–24 h). After removal from the gas chamber, cells were immediately processed for RNA extraction, or for reporter gene assay (see below).

RNA extraction and northern blot analysis

From cultured C6 and GS9L cells, total RNA was extracted with the Qiagen RNA isolation kit (RNeasy Total RNA kit, Qiagen, Hilden, Germany) according to the instructions of the manufacturer. From HUVEC and tumour tissues, total RNA was extracted according to the guanidinium thiocyanate method [4]. Aliquots of RNA (10–20 μg) were electrophoresed in a 1.5% agarose gel containing 15% formaldehyde and subsequently transferred to a Duralon membrane (Stratagene, La Jolla, USA) in $20 \times$ SSC. Filters were cross-linked with u.v. light (0.4 J/cm²) and hybridized at 68 °C in hybridization solution (QuickHyb, Stratagene, La Jolla, USA) or as described [16,29] with the following random primed ^{32}P-labelled cDNA probes: a full length 450 base-pair cDNA encoding for mouse VEGF164 [3], a 1.4-kb fragment encoding part of the extracellular domain of the KDR/VEGFR-2 gene [35]. For control of RNA loading we used a cDNA fragment coding for chicken β-actin.

The mRNA levels were quantified by densitometric analysis using Image Master System DTS (Pharmacia, Biotech). The expression indices of VEGF mRNAs were normalized against the β-actin value of each sample.

Conditioned media and Western analysis

After an incubation time of 16 h, the C6 or GS9L conditioned medium was removed, centrifuged for 10 min at 3000 r.p.m. to remove cells and filtered through a 0.22-μm filter (Millipore). The total cell count was determined in a Fuchs-Rosenthal chamber and cell viability was assessed by trypan blue staining. The conditioned medium was concentrated $\approx$10-fold using centricon with a 10-kDa cut-off (Amicon, Beverly, USA). Aliquots were separated on a 12.5% polyacrylamide-SDS gel and transferred to a PVDF membrane. The membrane was incubated with a polyclonal rabbit anti-VEGF antibody [3] directed against the N-terminal 16 amino acids of the mouse VEGF164 protein. A biotinylated goat antirabbit antibody was used as a secondary antibody (Dianova, Hamburg, Germany). After incubation with an streptavidin-biotinylated horseradish peroxidase complex (DAKO, Hamburg, Germany) visualization was performed by a chemoluminescence technique (ECL, Amersham International, Amersham, UK) and documented on a Kodak X-omat AR film (Kodak Ltd, Liverpool, UK).

Transplantation of GS9L cells

For *in vivo* experiments, syngeneic female rats (Fischer F344), weighing between 180 g and 200 g were purchased from Charles River, Germany. For subcutaneous transplantation, animals were briefly anaesthesized with ether. GS9L cells [106] suspended in 0.5 mL PBS were slowly injected into the right flanks. Animals were monitored daily for tumour growth. Fourteen days after tumour implantation, 2 mg dexamethasone or 0.9% saline was administered three times a day by i.p. injection.

Sixteen tumour-bearing rats were separated into two groups. Six rats each were treated with dexamethasone for 48 h and 72 h, respectively. For control purposes, four animals were injected with 0.2 mL 0.9% saline i.p. every 8 h. After 48 h and 72 h, two animals of the control group together with six animals of the treatment group were killed 4 h after the last injection. Tumours were removed, measured, weighted and divided into two parts. One part was frozen in liquid nitrogen for RNA analysis, whereas the other part was fixed in 4% paraformaldehyde, embedded in paraffin wax and processed for histological analysis.

Results

Dexamethasone but not progesterone or oestradiol downregulates VEGF expression in normoxic C6 and GS9L cells *in vitro*

We observed a dexamethasone-concentration-dependent downregulation of VEGF protein in the C6 cell conditioned medium. Whereas 1 nmol/l and 10 nmol/l dexamethasone had no significant effect on VEGF in the conditioned medium, 100 nmol/l and higher concentrations of dexamethasone led to a significant decrease of both secreted isoforms VEGF120 and VEGF 164 (Figure 1). Similar results were obtained in GS9L and MS mouse sarcoma cell lines (data not shown). We next determined whether this effect is specific for glucocorticoids and whether the glucocorticoid receptor is involved. When the glucocorticoid specific antagonist RU 43044 was added to C6 cells cultured in the presence of 10 μmol/l dexamethasone in an equimolar ratio, the expression of VEGF was similar to the level observed in the untreated control cells (Figure 2). This finding is consistent with the hypothesis that the effect of dexamethasone on VEGF expression is mediated by the glucocorticoid receptor. Because of the consistent and reproducible effect on VEGF expression seen with 1 μmol/l dexamethasone all subsequent *in vitro* experiments were carried out using this concentration.

As assessed by immunohistochemistry, unstimulated C6 cells showed a predominantly cytoplasmic localization of the glucocorticoid receptor *in vitro*. After stimulation with 1 mmol/l dexamethasone, a strong nuclear immunostaining was observed (data not shown). Immunofluorescence studies which were carried out on acetone-fixed frozen sections of transplanted C6 and GS9L gliomas also showed predominantly cytoplasmic staining in tumours of untreated animals (not shown), and thus confirmed the *in vitro* results. These observations are consistent with the hypothesis, that C6 and GS9L glioma cells express a functional glucocorticoid receptor. Consistent with the observed glucocorticoid-specific effect on VEGF expression, hydrocortisone but not progesterone or oestradiol inhibited VEGF expression in C6 glioma cells *in vitro* (Figure 2).

Dexamethasone downregulates VEGF mRNA expression in hypoxic C6 and GS9L cells to a lesser extent than in normoxic cells

To determine the influence of dexamethasone on VEGF mRNA levels Northern blot analyses were carried out using RNA prepared from C6 and GS9L cells cultured in the presence of 1 μmol/l dexamethasone. We observed 60% downregulation of VEGF mRNA in C6 cells ($P = 0.015$, five independent experiments), and 50% downregulation in GS9L cells ($P = 0.01$, three independent experiments) compared with untreated controls.

We next carried out a time course experiment by incubating C6 cells for 6 h, 12 h, 24 h, 48 h with 1 μmol/l dexamethasone, and observed a significant downregulation of VEGF mRNA after 6 h, which persisted for the various time points investigated (Figure 3).

An 8.2 fold (C6) and 7.3 fold (GS9L) upregulation of VEGF mRNA was observed after 16–24 h of hypoxia. This upregulation of VEGF mRNA with hypoxia was attenuated by dexamethasone to 7.1 fold (C6) and 5.2 fold (GS9L), so that, under hypoxic conditions, an inhibition of VEGF mRNA by dexamethasone of 13% in C6
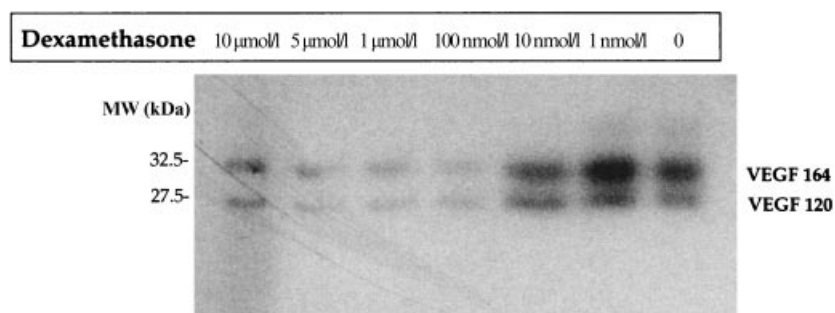

Figure 1. Concentration-dependent downregulation of VEGF protein by dexamethasone. Immunoblot analysis of conditioned media from C6 cells which were incubated with (1 nmol/l–10 μmol/l) or without (0) dexamethasone under normoxic conditions. Whereas 1 nmol/l and 10 nmol/l dexamethasone had no significant effect on VEGF in the conditioned medium, 100 nmol/l and higher concentrations led to a significant dowregulation of the secreted isoforms VEGF120 and VEGF164.
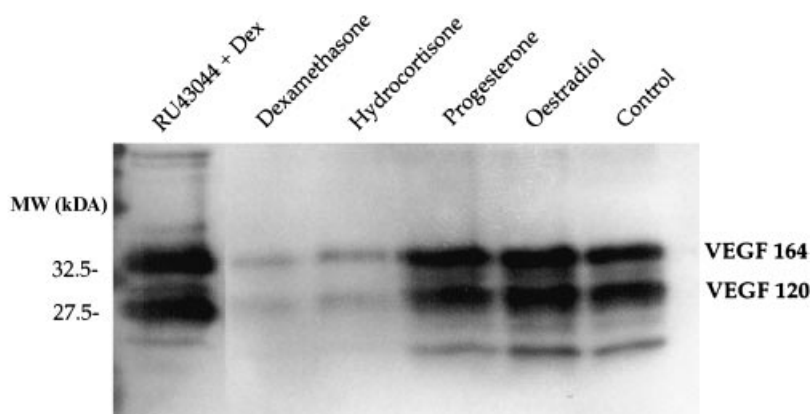
Figure 2. The effect of dexamethasone on VEGF expression is mediated by glucocorticoid receptors. Immunoblot analysis of the conditioned media from C6 cells incubated under normoxic conditions for 16 h with 10 μmol/l RU43044 and 10 μmol/l dexamethasone (Dex), 10 μmol/l dexamethasone, 10 μmol/l hydrocortisone, 10 μmol/l progesterone, 10 μmol/l oestradiol and without steroids. In the presence of an equimolar ratio of the glucocorticoid antagonist RU 43044 and dexamethasone, the expression of VEGF was similar to the level obeserved in the untreated control cells. Hydrocortisone but not progesterone or estradiol inhibited VEGF expression in C6 glioma cells.
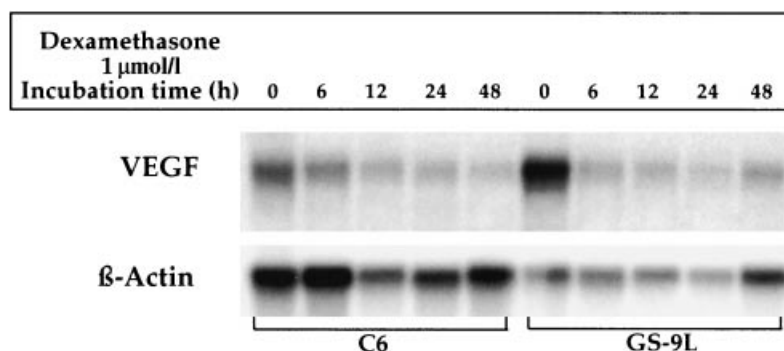

Figure 3. Effect of dexamethasone on VEGF mRNA. Northern blot analysis of 10 μg of total RNA derived from C6 and GS9L cells under normoxic conditions incubated with 1 μmol/l dexamethasone for 0 (control), 6, 12, 24 and 48 h. A significant downregulation of VEGF mRNA after 6 h was observed, which persisted for the various time points investigated.

($P=0.011$) was observed and of 30% in GS9L cells ($P=0.008$) compared with hypoxic cells without dexamethasone (Figure 4). Thus, compared with the 50–60% inhibition of VEGF mRNA expression observed under normoxic conditions, dexamethasone was significantly less potent in inhibiting VEGF mRNA expression under hypoxic conditions.

No significant downregulation of VEGF mRNA by dexamethasone in GS9L tumours *in vivo*

At the time of their removal, tumour weights were 820 mg (SD = 0.26) in the control group, 412 mg (SD = 0.05) in the dexamethasone group treated for 48 h and 432 mg (SD = 0.29) in the dexamethasone group treated for 72 h. Histological analysis revealed uniform tumours in all animals with typical spindle shaped cells. Microscopically, necrosis was present in large tumours.

Northern analysis showed VEGF mRNA expression in all tumours (data not shown). Compared with control animals, dexamethasone treated animals showed a 15% decrease in VEGF mRNA expression, which was, however, not statistically significant compared with controls ($P=0.24$). No difference was observed in VEGF mRNA expression between animals treated for 48 h or 72 h with dexamethasone.
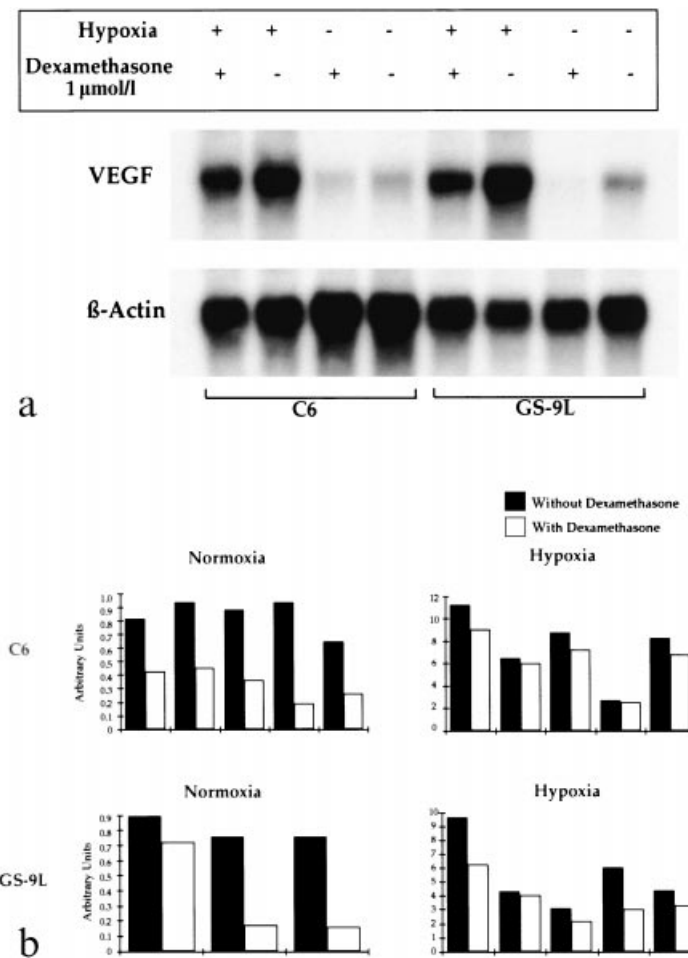
Figure 4. Dexamethasone downregulates VEGF mRNA expression in hypoxic C6 and GS9L cells to a lesser extent than in normoxic cells. **a,** Northern blot analysis: 10 µg of total RNA derived from C6 and GS9L cells under normoxia and hypoxia (16 h in anaerobic gas chamber), incubated with and without 1 µmol/l dexamethasone. **b,** VEGF mRNA relative amounts in normoxic and hypoxic C6 and GS9L cells treated (white bars) and untreated (black bars) with dexamethasone in independent experiments. The mRNA levels were quantified by densitometric analysis using an Image Master System DTS system (Pharmacia, Biotech). β-Actin was used as the loading control. The relative hybridization signal numbers were calculated by ascribing a value of 1 to an arbitrary signal.

Dexamethasone has no effect on VEGF receptor-2 (VEGFR-2/KDR/flk-1) expression in human umbilical vein endothelial cells (HUVEC) *in vitro* and in GS9L tumours *in vivo*

To determine whether dexamethasone exerts its effect on tumour vasculature integrity by regulating the expression of VEGF receptor-2, which is highly upregulated in tumour capillaries [25], we used HUVEC because these cells constitutively express VEGFR-2 [21]. Incubation of HUVEC with 1 or 10 µM dexamethasone for 16 h did not affect the mRNA level of the signal-transducing VEGF receptor-2 (Figure 5). To examine an

effect of dexamethasone on VEGF receptor-2 expression *in vivo*, we analysed RNA extracted from dexamethasone treated and from control tumours by Northern analysis. Consistent with the *in vitro* observations, we found a similar VEGF receptor-2 mRNA expression in dexamethasone-treated and in untreated control GS9L tumours.

Discussion

We tested the hypothesis that the antioedema effect of dexamethasone is mediated by downregulation of VEGF or VEGF receptor expression. Using two glial rat tumour cell lines we observed a strong downregulation of VEGF
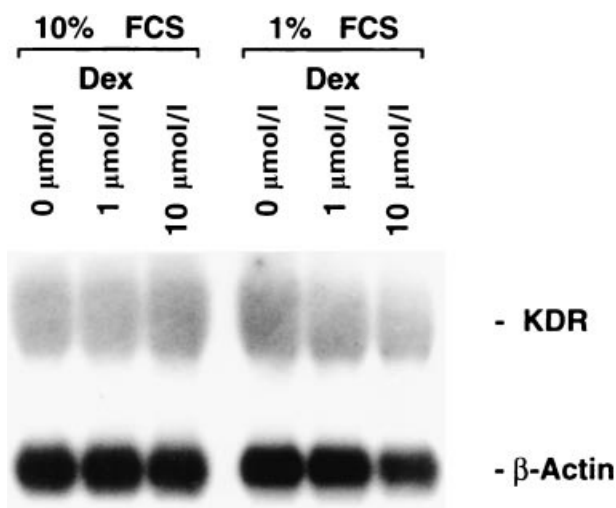
Figure 5. Dexamethasone has no effect on VEGF receptor-2 (VEGFR-2/KDR/flk-1) expression in human umbilical vein endothelial cells (HUVEC). Northern analysis of KDR/VEGFR-2 expression in HUVE cells. Dex, Dexamethasone; FCS, Fetal calf serum.

mRNA and protein expression was observed following incubation of cells with dexamethasone. This effect was dependent upon concentration. A significant downregulation of VEGF protein expression was seen at concentrations of 100 nmol/l dexamethasone or higher. Since VEGF downregulation could also be achieved by hydrocortisone, but not by progesterone or oestradiol, our results suggest a glucocorticoid receptor-specific downregulation of VEGF in the glioma cell lines used. This is further supported by our observation that downregulation of VEGF by dexamethasone was completely reversed by coincubation with the glucocorticoid specific antagonist RU 43443. In line with these findings, we observed expression of the glucocorticoid receptor in C6 glioma cells *in vitro* and *in vivo* and nuclear translocation of the ligand-receptor complex after addition of dexamethasone *in vitro*, suggesting that C6 cells possess a functional glucocorticoid receptor. These findings support the hypothesis that the dexamethasone effect on VEGF expression is mediated by the glucocorticoid receptor.

Hypoxia has been shown to be a major inducer of VEGF expression *in vitro* [25,32]. Ikeda *et al.* demonstrated that VEGF upregulation in glioma cells under hypoxia is due to both an increase in mRNA stability and transcriptional activation [16]. Transcriptional activation of the VEGF gene is mainly achieved by binding

of a hypoxia-inducible transcription factor (HIF-1α) to the hypoxia-responsive element (HRE) in the VEGF promoter [11]. In contrast, VEGF mRNA stability is under control of hypoxia-regulated sequences in the 3′UTR of the VEGF mRNA [23]. Transcriptional activation and mRNA stability contribute to the upregulation of VEGF expression at different timepoints. Hypoxia leads to an early (e.g. after 3 h) transcriptional activation of the VEGF gene in C6 glioma cells with an two- to threefold increase in transcription rate. After 15 h of hypoxia, increase in VEGF message is partly due to increased transcription (two- to threefold) and partly due to increased mRNA stabilization (two- to threefold) [16,23]. We therefore investigated whether dexamethasone inhibits VEGF expression under both normoxic and under hypoxic conditions in C6 and GS9L cells. *In vitro*, dexamethasone-induced downregulation of VEGF was significantly higher in normoxic (50% in C6 and 60% in GS9L cells) than in hypoxic glioma cells (13% in C6 and 30% in GS9L cells). It is unclear why dexamethasone downregulates VEGF to a far greater extent in normoxic than in hypoxic glioma cells. The VEGF gene contains several binding sites for the transcription factor AP1 [36]. The dexamethasone-glucocorticoid receptor complex prevents the AP1 transactivator complex from stimulating transcription of genes containing an AP1 binding site [18]. This has also been shown for the VEGF gene [10]. Possibly dexamethasone inhibits AP1-dependent transcription in the VEGF gene, but leaves the activity of hypoxia-dependent transcription factors, such as HIF-1α, unaltered. In addition, dexamethasone may interfere to some extent with the increase in VEGF mRNA stability observed under hypoxic conditions.

We also wanted to determine whether VEGF is downregulated by dexamethasone in gliomas *in vivo.* For these experiments, GS9L cells were injected subcutaneously into syngeneic rats. Animals were treated with 6 mg dexamethasone per day for 48 and 72 hours, respectively. By Northern analysis, we observed no significant (e.g. 15%, $P = 0.24$) downregulation of VEGF in dexamethasone-treated tumours compared with controls. Although there are several possible explanations for the reduced potency of dexamethasone to inhibit VEGF expression *in vivo,* the most likely is that *in vivo* a considerable proportion of tumour cells suffer from hypoxia. The tumours we analysed well exceeded the diameter of 2 mm which, based on experimental models,

has been proposed as being critical for angiogenesis-independent vs angiogenesis-dependent tumour growth. It has been shown by the use of the nitroimidazole compound EF5, that GS9L tumours are hypoxic *in vivo* [8,9]. Thus, we assume that VEGF downregulation by dexamethasone *in vivo* was largely prevented in hypoxic tumour cells. Other possibilities to explain the discrepancies observed *in vitro* and *in vivo* may include an insufficient dosage of dexamethasone used *in vivo*. However, in the animal experiments, we used dexamethasone doses well above the concentrations used *in vitro*. Since these doses exert an antioedema effect on rats with intracranial tumours [27], it is unlikely that the doses of dexamethasone used *in vivo* were insufficient. It also appears unlikely that we chose an inappropriate time point for tumour excision and evaluation of VEGF expression since our *in vitro* results demonstrated a prolonged (e.g. up to 72 h) effect of dexamethasone on VEGF expression.

Finally we tested the hypothesis, that dexamethasone influences peritumoural oedema by the downregulation of VEGF-receptors. In this scenario, VEGF produced by tumour cells would fail to induce vascular permeability due to lack of receptors on endothelial cells. Current evidence suggests that VEGFR-2, but not VEGFR-1, mediates vascular permeability in endothelial cells *in vitro* and *in vivo*. We therefore examined the effect of dexamethasone on VEGFR-2 expression. No effect of dexamethasone on VEGF receptor-2 mRNA expression in human umbilical vein endothelial cells *in vitro* nor on GS9L tumours *in vivo* was observed. However, VEGFR-2 protein regulation by dexamethasone at a post-translational level or functional inhibition of the receptor cannot be excluded.

The present results confirm and extend a study by Heiss *et al.* who observed 70% suppression of vascular permeability inducing activity by dexamethasone in glioma conditioned medium and downregulation of VEGF mRNA in glioma cells [14]. We observed in addition, that dexamethasone downregulates the expression of VEGF/VPF in glioma cells far less efficiently *in vivo* than *in vitro*. Our studies suggest, that this discrepancy is most likely due to induction, by hypoxia, of VEGF expression in glioma cells *in vivo*. These observations are consistent with the hypothesis that, in human malignant gliomas, the downregulation of VEGF underlies the antioedema effect of dexamethasone, but suggest that the efficacy of dexamethasone *in vivo* is limited by tumour hypoxia.

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