Autocrine regulation of glioblastoma cell-cycle progression, viability and radioresistance through the VEGF-VEGFR2 (KDR) interplay

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To cite this article: Petra Knizetova, Jiri Ehrmann, Alice Hlobilkova, Iveta Vancova, Ondrej Kalita, Zdenek Kolar & Jiri Bartek (2008) Autocrine regulation of glioblastoma cell-cycle progression, viability and radioresistance through the VEGF-VEGFR2 (KDR) interplay, Cell Cycle, 7:16, 2553-2561, DOI: 10.4161/cc.7.16.6442

To link to this article: http://dx.doi.org/10.4161/cc.7.16.6442

Published online: 06 Aug 2008.

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Vascular endothelial growth factor (VEGF) plays a crucial role in angiogenesis and progression of malignant brain tumors. Given the significance of tumor microenvironment in general, and the established role of paracrine VEGF signaling in glioblastoma (GBM) biology in particular, we explored the potential autocrine control of human astrocytoma behavior by VEGF. Using a range of cell and molecular biology approaches to study a panel of astrocytoma (grade III and IV/GBM)-derived cell lines and a series of clinical specimens from low- and high-grade astrocytomas, we show that co-expression of VEGF and VEGF receptors (VEGFRs) occurs commonly in astrocytoma cells. We found VEGF secretion and VEGF-induced biological effects (modulation of cell cycle progression and enhanced viability of glioblastoma cells) to function in an autocrine manner. Moreover, we demonstrated that the autocrine VEGF signaling is mediated via VEGFR2 (KDR), and involves co-activation of the c-Raf/MAPK, PI3K/Akt and PLC/PKC pathways. Blockade of VEGFR2 by the selective inhibitor (SU1498) abrogated the VEGF-mediated enhancement of astrocytoma cell proliferation and viability under unperturbed culture conditions. In addition, such interference with VEGF-VEGFR2 signaling potentiated the ionizing radiation-induced tumor cell death. In clinical specimens, both VEGFRs and VEGF were co-expressed in astroglial tumor cells, and higher VEGF expression correlated with tumor progression, thereby supporting the relevance of functional VEGF-VEGFR signaling in vivo. Overall, our results are consistent with a potential autocrine role of the VEGF-VEGFR2 (KDR) interplay as a factor contributing to malignant astrocytoma growth and radioresistance, thereby supporting the candidacy of this signaling cascade as a therapeutic target, possibly in combination with radiotherapy.

Abbreviations: VEGF, vascular endothelial growth factor; GBM, glioblastoma multiforme; VEGFR, vascular endothelial growth factor receptor; KDR, kinase-insert domain receptor; Flt-1, fms-related tyrosine kinase-1; LGA, low grade astrocytoma; HGA, high grade astrocytoma; IR, ionizing radiation; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PKC, protein kinase C; PI, propidium iodide; ELISA, enzyme linked immunosorbent assay

Key words: astrocytoma, glioblastoma, vascular endothelial growth factor, cell cycle, autocrine signaling, ionizing radiation, cell survival

Introduction

Interactions between tumor cells and their microenvironment are critically important in the biology of cancer, and include growth factor signaling in paracrine and autocrine manners.\(^1\)\(^-\)\(^5\) Besides its pro-angiogenic function, VEGF also increases vascular permeability and VEGF overexpression occurs in a variety of tumors including highly vascularized and infiltrative astrocytomas (grade III/IV), where VEGF expression correlates with poor prognosis.\(^6\)\(^-\)\(^7\) The bulk of current knowledge about the biology of VEGF and VEGFRs derives from studies of endothelial cells and VEGF’s paracrine effects when secreted by diverse tumor cell types. Emerging evidence, however, suggests co-expression of VEGF and VEGFRs in some types of tumors and highlights the importance of understanding the potential autocrine loop-signaling of VEGF within a tumor mass. Moreover, it has been observed that serum VEGF levels are elevated in subsets of patients with malignant tumors after ionizing radiation (IR) therapy.\(^8\) These findings suggest that the VEGF-mediated paracrine signaling between brain tumor mass and its vasculature, and possibly autocrine effects of VEGF on tumor growth and cell viability, may represent a plausible target for a novel strategy to sensitize malignant astrocytomas to radiation treatment.

Despite the well established role of angiogenesis and tumor spread in malignant astrocytomas, however, very little is known about the autocrine effect(s) of VEGF in human astrocytoma biology and clinical behavior. To address this issue, the present study was designed to assess the potential autocrine role of VEGF signaling in astrocytomas, using a range of molecular and cell biology approaches to examine a panel of human astrocytoma (mainly GBM) cell lines, complemented by immunohistochemical analyses of VEGF and its two receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR), in a series of clinical specimens from low- and high-grade human astrocytomas. As described below, our results not only support such plausible autocrine regulation of astrocytomas by VEGF, but also provide novel insights into the critical VEGF receptor type and the downstream intracellular signaling pathways involved. Finally, our study shows an...
anti-tumor activity of a small molecule capable of inhibiting the VEGF signaling cascade in astrocytoma cell lines, and combined experiments with IR support a scientific rationale for effective use of a targeted anti-VEGF(R) therapy in combination with a DNA-damaging treatment such as IR.

**Results**

Co-expression of VEGF and its two receptors in human astrocytoma cell lines. To explore possible signal transduction pathways for VEGF, expression of VEGF and VEGFRs: Flt-1 (VEGFR1) and KDR (VEGFR2) (Fig. 1A) was analyzed using RT-PCR in a panel of eight human astrocytoma/GBM cell lines. VEGF, Flt-1 and KDR mRNAs were expressed in all the cell lines tested (Fig. 1B). Endothelial cells (HUVEC) known to express both Flt-1 and KDR and SY-5Y neuroblastoma cells known to lack expression of VEGFRs served as positive and negative controls, respectively.9,10 The RT-PCR analysis of HUVEC cells yielded products of the expected size. The VEGF primers were designed within exon 2 and the 3' untranslated region of the VEGF cDNA, thereby allowing amplification of all known VEGF splice variants in a single reaction.11 We were able to amplify cDNAs for three VEGF isoforms: VEGF<sub>121</sub>, VEGF<sub>165</sub> and VEGF<sub>189</sub>, which are known to be expressed in astrocytoma cell lines, with the VEGF<sub>121</sub> isoform being expressed at the highest level (Fig. 1C).

VEGF signaling in astrocytoma cells is mediated via VEGFR2 (KDR). To gain a better insight into the functional significance of VEGF-VEGFR co-expression, we first sought to assess the cellular effects of VEGF signaling by Western blot analysis of the phosphorylation status of key components of the three major signaling pathways implicated in cell growth, proliferation, migration and survival: the PI3-K/Akt, c-Raf/MAPK and PLCγ1/PKC cascades, respectively. VEGF<sub>121</sub> (20 ng/ml) added to cultured human A172 glioblastoma cells stimulated phosphorylation of c-Raf (Ser338), MAPK (Thr202/Tyr204), Akt (Thr308) and PLCγ1 (Tyr783) in a time-dependent manner (Fig. 2A), reaching the maximum at 30–60 minutes of treatment. In addition, these results were not restricted to A172 cells, as similar data were obtained when the same treatment with VEGF<sub>121</sub> was applied to another astroglial tumor cell line, U251MG (data not shown). The observed responses were transient, and by 12 hours of VEGF<sub>121</sub> stimulation, the degree of phosphorylations in the majority of the monitored proteins returned to basal levels (Fig. 2A).

Next, we analyzed the ability of two ligands of VEGFRs: PlGF-1 and VEGF<sub>121</sub>, and the small molecule inhibitor of VEGFR2/KDR: SU1498, to stimulate or inhibit functional VEGF signaling, respectively. While VEGF<sub>121</sub> is capable of stimulating both VEGF receptors, PlGF-1 is a known selective ligand for VEGFR1 only, and SU1498 represents a highly specific VEGFR2 tyrosine kinase domain inhibitor.12,13 To judge the effects of these different treatments on intracellular VEGF signaling in glioblastoma cells, selected markers including phospho-Akt (Ser473) and phospho-p44/42 MAPK (Thr202/Tyr204) were monitored by Western blotting. As shown in Figure 2B, VEGF<sub>121</sub> but not PlGF-1 was able to induce phosphorylation of Akt/PKB and p44/42 MAPK when either ligand was applied at an identical concentration (20 ng/ml). Interestingly, pre-treatment of the glioblastoma cells with the VEGFR2 tyrosine kinase inhibitor SU1498 (30 μM, applied one hour before VEGF treatment) abrogated the otherwise pronounced stimulatory effect of VEGF<sub>121</sub> on Akt (Ser473) and p44/42-MAPK (Thr202/Tyr204).
phosphorylation levels (Fig. 2B). Collectively, these results strongly indicate that the key receptor through which the signaling pathways are triggered by VEGF in our GBM cell model is KDR (VEGFR2).

**Exogenous VEGF stimulates endogenous VEGF mRNA levels and VEGF secretion in cultured glioblastoma cells.** If an autocrine loop of VEGF signaling should indeed operate in astrocytomas, another strong prediction would be that VEGF itself should be secreted by the tumor cells, and possibly regulate also the expression of endogenous VEGF production. The latter effect has been reported for endothelial cells, but there is no information on VEGF-mediated regulation of VEGF expression in astrocytomas. To test these conceptually important issues, we first set to determine whether recombinant VEGF121 is able to induce alterations of endogenous VEGF expression levels in the GBM cell line A172, as assessed by semi-quantitative RT-PCR. Compared to a mock-treated control sample, VEGF mRNA levels increased by 6–12 hours of treatment in the VEGF-exposed cells (Fig. 3A), and this effect persisted for the next 24–48 hours.

To investigate whether the observed increase in VEGF mRNA is accompanied by enhanced VEGF protein secretion, we used ELISA to examine the effect of exogenous VEGF121 (40 ng/ml) on VEGF secretion by the A172 cells (Fig. 3B). Under serum-free conditions, when cells were washed 2 hours after VEGF stimulation, a significant secretion of VEGF, increasing over the time, was detected. Importantly, this effect was durable, as documented by elevated VEGF secretion for up to 48 hours after the 2-hour pulse-stimulation with VEGF121. In addition, when the signaling of the added exogenous VEGF121 into the cells was blocked by addition of the VEGFR2 kinase inhibitor SU1498 (30 μM, for 1 hour), the VEGF-dependent increase of the secreted endogenous VEGF was inhibited (Fig. 3C). This critical result documents that the extracellular VEGF, which we detected at increasing amounts with time after washing out the initially added exogenous VEGF, was indeed secreted (rather than representing a fraction of the exogenous VEGF still adherent to cell membrane throughout the washing period and then being released over time). Furthermore, this experiment also indicates that the autocrine loop signaling of VEGF is mediated via the VEGFR2 (KDR) receptor. Our results show that astrocytoma cells are capable of responding to VEGF stimulation by enhanced expression and secretion of VEGF into their extracellular environment, a notion consistent with the potential autocrine role of VEGF.

VEGF stimulates cell growth of glioblastoma cell lines. To examine the biological impact of VEGF stimulation on human GBM cell models, we treated the cell lines A172 and U251MG with increasing concentrations of VEGF (10, 20, 40 and 80 ng/ml) and measured MTT absorbance in time-course experiments, as an index of cell growth/proliferation, as employed in previous studies of VEGF-induced astrocytoma cell proliferation. As early as 6–12 hours after addition of VEGF121 to culture medium, MTT absorbance was significantly increased (by 19–35% for A172 and 14–79% for U251MG, p < 0.001 for any of the four VEGF concentrations used) compared with the mock-treated control cells. Despite some modestly higher increases of MTT absorbance at the higher versus lower concentrations of added VEGF, such concentration-dependence was not statistically significant. Unlike exposure to VEGF, treatment of the same GBM cell lines with the VEGFR1-selective ligand PlGF-1 did not induce any detectable increase of the MTT absorbance (negative data, not shown). Importantly, selective blockade of the VEGFR2 (KDR) receptor by SU1498 pre-treatment prevented the VEGF-dependent increase of cell growth as judged from the decreased MTT absorbance at 6, 12, 24 and 48 hours after VEGF addition (p < 0.001 at all time-points, data not shown).

To examine whether the above effects of VEGF on cell growth/viability were accompanied by increased entry into S phase and DNA synthesis, incorporation of EdU (equivalent of BrdU, after 30-minute pulse-labeling with EdU at 6, 12 and 24 hours after VEGF addition) in the two serum-starved GBM cell lines was assessed. As shown...
in Figure 4A and B, VEGF stimulation led to a significant increase in the proportion of EdU-positive cells in both the A172 and U251MG cell lines, with maximum at 6–12 hours of treatment. Interestingly, pre-treatment of either cell line with the SU1498 inhibitor of VEGF2/KDR abrogated the VEGF-induced effect, and by 24 hours of incubation the number of proliferating cells (cells incorporating EdU) dropped below even the control levels seen in the VEGF-unstimulated cells (Fig. 4A and B). These results document a positive impact of VEGF on cell growth/metabolic activity and cell cycle progression (S-phase entry) in two astrocytoma models, and suggest that these biological effects are critically dependent on intact signaling through the VEGF2 (KDR) receptor.

**VEGF as a radiation-protective factor in glioblastoma cells.** IR represents a key therapeutic modality for malignant astrocytomas,19 VEGF is elevated in astroglial tumor cell lines after IR,15,18 and it has recently been proposed that VEGF might protect tumor blood vessels from IR-induced cytotoxicity and thereby possibly contribute to tumor radioreistance in a paracrine manner.20 These facts, and our present data (see above) prompted us to explore a possible role of the autocrine VEGF signaling in the response of malignant astrocytoma models to IR. To this end, GBM cell lines A172 and U251MG were serum starved for 24 hours, treated with SU1498 (30 μM) or left untreated, and 2 hours later the cells were either mock-treated or irradiated with 10 Gy of IR. During the next 24 hours, cells were grown under low-serum [1% (v/v) FBS) conditions and apoptosis was examined 24 hours post-irradiation. The key results from these experiments were the following: (i) Treatment with SU1498 alone undermined the viability of the astrocytoma model cell lines, leading to enhanced apoptosis in both A172 and U251MG cells (Fig. 5A and B); (ii) Radiation alone induced pronounced apoptosis of A172 cells, while the U251MG cell line proved resistant to IR (Fig. 5A and B); (iii) Most importantly, the combined treatment with SU1498 and IR clearly further potentiated cell death, leading to increased apoptosis above the additive values in both cell lines, including the otherwise IR-resistant U251MG cells (Fig. 5A and B).

Overall, these results suggest that VEGF can play a “neuro-protective” role in astrocytoma cells, at least in vitro, and that inhibition of the VEGF-VEGF2/KDR signaling cascade sensitizes human GBM cell lines to cytotoxic effects of ionizing radiation treatment.

**VEGF expression is related to astrocytoma progression in clinical specimens.** Since our data obtained with the cell culture models were consistent with a potential autocrine role of VEGF-VEGF2 signaling in regulation of cell cycle and viability of human astrocytomas, we set to search for a biological correlate of such a role directly in clinical specimens from human astroglial tumors of diverse malignant potential (grade of progression). Two questions were addressed by immunohistochemical analysis of VEGF, and the two VEGF receptors on tissue sections: (i) Whether or not VEGF and VEGFRs are coexpressed in such tumors, as would be predicted for an autocrine loop of VEGF signaling; and (ii) Whether there is any correlation between the levels of VEGF and/or VEGFRs and clinical progression of the tumors.

In contrast to control human tissues that scored negative (data not shown), immunoreactivity for VEGF was found in all astroglial tumors irrespective of tumor grade, with variable (commonly strong) staining intensity and heterogeneous pattern of positivity (Fig. 6). Overall, statistically significant higher VEGF expression was found among the series of 35 high-grade astrocytomas (HGA) when compared to the 28 specimens from low-grade astrocytomas (LGA, p = 0.026; cut off value of the semi-quantitative histoscore values: 90). On the other hand, despite a modest trend towards higher expression of the VEGF receptors in HGA, the observed differences between lesions of different grades were not statistically significant (VEGF1/Fit-1: histoscore values 47.1 and 43.4 in HGA and LGA, respectively; and for VEGFR2/KDR: histoscore values 84.2 and 74.3 in HGA and LGA, respectively). In summary, these immunohistochemical results confirmed co-expression of VEGF and its cognate
receptors on astrocytoma tumor cells directly in clinical material, and showed a positive correlation between VEGF abundance and astroglial tumor progression in vivo.

**Discussion**

Malignant astrocytomas and especially glioblastoma multiforme (GBM) are aggressive tumors characterized by rich angiogenesis, the critical role of which is documented by the ability of anti-angiogenic inhibitors to decrease tumor growth in vivo.\(^{21}\) Previous studies have also suggested that overexpression of VEGF in malignant astrocytomas is associated with increased vascular density and poor clinical outcome.\(^{23,24}\) However, discrepant findings on biological function(s) of VEGF and its contribution to astrocytoma progression have been reported,\(^{15-18}\) raising the need to clarify the nature and significance of potential VEGF-mediated autocrine signaling in astroglial tumors.

In the present study, we provide evidence for a VEGF-mediated autocrine regulatory loop in human GBM cells, and document several biological effects of VEGF, including radioprotection. The predictions for autocrine signaling of VEGF, that were tested and indeed experimentally validated by our work include the following: (i) VEGF is expressed by astroglial tumor cells in a panel of human cell lines as well as in clinical specimens, as documented at the mRNA and/or protein levels; (ii) The receptors for VEGF are commonly co-expressed by human astroglial tumor cells in both the in vitro and clinical (in vivo) scenarios; (iii) Expression of endogenous VEGF mRNA is enhanced, and secretion of endogenous VEGF into extracellular environment is induced, upon VEGF-mediated stimulation of cultured human GBM cells; (iv) Exposure to physiological concentrations of VEGF elicits a host of biological responses in GBM cells, including effects on cell growth, cell cycle progression and enhanced viability; (v) Increasing abundance of VEGF correlates with progression of human astrocytomas from low- to high-grade tumors.

Apart from the evidence for autocrine signaling, our data also provide some insights into the preferential VEGF receptor type and the intracellular pathways involved in the response of astroglial tumor cells to VEGF. Whereas both VEGFR1 (Flt-1) and VEGFR2 (KDR) are expressed in the astroglial tumor cell lines and tumors in vivo, the lack of the VEGFR1-selective ligand PlGF-1 to induce a detectable response, and especially the ability of the selective VEGFR2 inhibitor SU1498 to counteract the VEGF biological effects, indicate a dominant role of VEGFR2 (KDR) in the mechanism of VEGF signaling in our GBM models. Our data do not exclude a possible role of VEGFR1 in subsets of astrocytomas, however the observed preferential signaling via VEGFR2 suggests that VEGFR2 (KDR) could represent a promising target for inhibition of astroglial tumor growth.

Extending the current knowledge about the known multifaceted signaling network triggered by VEGF in other cell types, particularly in endothelial cells,\(^{25-28}\) our present analysis revealed the involvement of at least three pathways: the c-Raf/MAPK, PI3-K/Akt and PLC\(\gamma\)/PKC cascades, in VEGF-stimulated astroglial tumor cells. The activation of these pathways was monitored by induced phosphorylation events on key proteins within these signaling cascades: c-Raf/MAPK, PI3-K/Akt and PLC\(\gamma\), respectively. The kinetics of these phosphorylations was consistent with a prompt multi-pathway response to VEGF in the model A172 cell line. One exception we observed was the constitutively rather high level of phosphorylation at Akt Ser473 in our experiments, likely attributable to a loss-of-function mutation of the tumor suppressor gene PTEN in the A172 cells.\(^{29}\) PTEN is a strong negative regulator of the PI3K/Akt pathway, and hence the absence of this regulatory effect could result in high basal phospho-Akt Ser473 levels in this cell line.\(^{29}\) Activated Akt phosphorylates numerous downstream effectors to modify important cellular processes and, at least in endothelial cells, the PI3K/Akt axis promotes cell viability, thereby controlling the balance between survival and cell death.\(^{30}\) Our biochemical and cell biology data indicate that in human astrocytoma cells, VEGF
triggers multiple interacting pathways and, as discussed in the next section below, such signaling translates into cell fate decisions about cell cycle progression and cell survival.

From a biological perspective, our experiments showed VEGF-induced effects in terms of promoted cell growth and cell cycle progression of the two model glioblastoma cell lines, as monitored by the enhanced metabolic activity (MTT test) and accelerated S-phase entry (EdU incorporation during DNA replication), respectively. While these effects were significant, they were relatively modest and transient. In contrast, the arguably most noticeable biological effect was the modulation of cell viability, both in unperturbed growth conditions and even more so after exposure to ionizing radiation. Our experiments with blocking the VEGF-VEGFR2 (KDR) signaling via a small molecule chemical inhibitor SU1498 indicate that the two GBM cell lines tested, A172 and U251MG, both require ongoing VEGF-mediated signaling for optimal viability. This is particularly noteworthy in the case of the p53-mutant U251MG cells that proved to be radioresistant, while responding by enhanced apoptosis when their VEGFR2 (KDR)-mediated signaling was blocked under standard growth conditions. These results with the GBM cell lines were cell-type specific, since analogous exposure of normal human fibroblasts to SU1498 did not cause any cytotoxicity (our unpublished data), further indicating that interference with VEGF signaling could be a promising strategy for treatment of astrocytomas including GBM.

Ionizing radiation is a mainstream treatment modality for malignant astrocytomas, and the inherent or progressively acquired resistance to such DNA-damaging treatment is a major cause of overall treatment failure and dismal prognosis of these brain malignancies. In this regard, we believe that our present data on the cooperative effects of IR and concomitant blocking of VEGFR2 signaling is potentially very relevant to astrocytoma behavior and response to therapy. Importantly, the concomitant treatment with IR and SU1498 resulted in a synergistic, rather than just additive or overlapping effect, indicating that VEGF signaling provides some kind of radioprotective function to astrocytoma cells, and this can be overcome by blocking the VEGFR2 signaling axis (Fig. 7).

Considered within the context of astrocytoma biology and response to DNA damaging treatments such as IR, it will now be important to pinpoint the nature of such radioprotective effect of VEGF signaling, and pursue the tempting possibility to exploit the observed synergistic effect for a more efficient, combined targeted therapy in a preclinical setting. As to the underlying mechanisms, one might speculate that the VEGF-triggered pathways not only affect the cell death machinery, but could also impinge on the DNA damage response pathways, either by modulating the checkpoint responses or efficiency of DNA repair, both of which are critical for the biological outcome and are often deregulated in human cancer. Another conceptually important issue is whether the so-called cancer stem cells, recently identified also in malignant astrocytomas, might
be targeted by such combined treatment. Among other features, these astrocytoma stem-like cells are particularly resistant to IR, and they have been shown to respond favorably to inhibition of DNA damage checkpoint or repair, and standard DNA damaging modality such as IR or chemotherapy, and data on the VEGF-VEGFR2 (KDR) signaling, would be to apply a domain inhibitor, SU1498.

**Materials and Methods**

**Cell culture.** Human astroglial tumor (grade III and IV-GBM) cell lines U118MG, U87MG, T98G, A172, Gli-06, SF126, U373MG (the latter three cell lines provided by C.Y. Bree, University of Amsterdam, The Netherlands) and U251MG, and the control HUVEC cells were maintained in Dulbecco's Eagle's medium (DMEM, standard medium) supplemented with 10% (v/v) fetal bovine serum, 1.7 mM L-glutamine and penicillin/streptomycin. The neuroblastoma cell line SY-5Y (ATTC) was maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 1.7 mM L-glutamine and penicillin/streptomycin. HUVEC cells were provided by J. Prochazkova (Institute of Biophysics, Brno, Czech Republic).

**Total RNA isolation and RT-PCR analysis.** Total RNA (2 μg) from astroglial tumor cell lines (U118MG, U87MG, T98G, A172, Gli-06, SF126, U373MG, U251MG), neuroblastoma cell line (SY-5Y) and HUVEC cells was extracted with TRIZOL® (Invitrogen) and reverse transcribed using human-specific primers for VEGF, Flt-1, KDR, and primers for GAPDH used as a RNA quality control. Primers for VEGF, Flt-1 and KDR were designed, and conditions for RT-PCR reactions set as published previously.

**Semi-quantitative RT-PCR analysis.** Total RNA (600 ng) was isolated from the cell line A172, serum-starved for 24 hours, then either left unstimulated or stimulated with recombinant VEGF$_{121}$ (20 ng/ml) for 6, 12, 24 and 48 hours, followed by semi-quantitative RT-PCR analysis for VEGF mRNA expression using GAPDH as a house-keeping gene control.

**ELISA.** Cells were serum-starved for 24 hours, either pre-treated by the SU1498 inhibitor (30 μM; from Calbiochem) for one hour, followed by stimulation with 40 ng/ml of VEGF$_{121}$, or incubated in medium containing 40 ng/ml of VEGF$_{121}$ only. Negative control cells were left untreated. Following 2 hours of stimulation, cells were washed twice with PBS and supplemented with serum-free medium and allowed to secrete VEGF for 6, 12, 24 and 48 hours. Secretion of VEGF into cell culture supernatants was measured by Human VEGF Quantikine Kit (R&D Systems) according to manufacturer's instructions. All tests were performed 3 times in triplicate.

**Western blot analysis.** The 6 cm dishes were seeded with 300,000 A172 cells in standard medium (see above) and cultured for 24 hours at 37°C in humidified atmosphere with 5% CO$_2$. Following 24 hours of starvation, cells were stimulated for 0.5, 1, 6 and 12 hours with 20 ng/ml of recombinant VEGF$_{121}$ (Sigma) or 20 ng/ml PlGF-1 (Chemicon). When using SU1498 (KDR inhibitor, 30 μM, Calbiochem), cells were pre-incubated with the inhibitor for 1 hour before stimulation with VEGF$_{121}$. Dishes with un-treated and treated cells were washed twice with ice-cold PBS and exposed to 120 μl of lysis buffer [containing 150 mM NaCl, 1% (v/v) NP-40, 50 mM Tris, pH 8.0, 0.5 mM EDTA, protease inhibitor cocktail tablets Complete (Roche Diagnostics)] at time points 0.5 h, 1 h, 6 h and 12 h after VEGF addition. The lysate was centrifuged (15,000 rpm) for 30 minutes at 4°C, and the supernatant was kept and stored at -80°C until use. Total protein content per sample was analyzed by Bradford protein assay (Bio-Rad, Hercules, CA). Protein loading buffer 4x concentrated (200 mM Tris-HCL, pH 7.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, and 40% glycerol) was added to each sample and boiled at 95°C for 4 minutes. Equal amounts (40 μg) of total protein extracted from cells were subjected to 10% SDS-PAGE. Then proteins were transferred using semidry Trans-Blot SD cell (Bio-Rad) onto a nitrocellulose membrane Hybrid-ECL (Amersham Biosciences). Membranes were blocked in 5% (w/v) fat milk in PBS containing 0.1% Tween-20 and incubated with primary antibodies (phospho-Akt Ser473, phospho-c-Raf Ser338, phospho-p44/p42 MAPK Thr202/Tyr204, phospho-PLCγ1 Tyr783, Cell Signaling Technology) overnight at 4°C. Finally, the membranes were rinsed with PBS containing 0.1% Tween-20 for 45 minutes and incubated with secondary antibodies (anti-mouse/anti-rabbit IgG, HRP-linked antibody, Cell Signaling Technology) at room temperature for 45 minutes, washed in PBS with 0.1% Tween-20 for 45 minutes, and the reaction visualized using the ECL solution (Amersham Biosciences).

**Click-iTTM EdU Alexa Fluor 488 Cell Proliferation Assay.** Following 24 hours of starvation in serum-free medium, cells were incubated with VEGF$_{121}$ (40 ng/ml) or pre-treated with SU1498 (30 μM) or...

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**Figure 7.** Model of autocrine regulation of VEGF signaling in human astrocytomas. We propose that regulation and biological effects of VEGF in VEGF-expressing astrocytomas including GBM operate, at least in part, in an autocrine manner. Signaling is mediated by VEGFR2 (KDR) and activates multiple intracellular pathways, as indicated. Beside stimulation of cell growth/proliferation, the pool of VEGF secreted by astrocytoma cells within the stromal niche creates a "pro-survival microenvironment" that enhances the viability of the tumor cells and protects them against IR-induced apoptosis. Autocrine (**) VEGF intracellular signaling as well as its biological effects may be inhibited by pre-treatment with the selective KDR tyrosine kinase domain inhibitor, SU1498.
left untreated for 6, 12 and 24 hours. Next, cells were pulse-labeled with 10 μM EdU for 30 min, harvested and analyzed according to manufacturer’s protocol for the Click-iT™ EdU Alexa Fluor 488 Cell Proliferation Kit (Invitrogen). Experiments were performed 3 times in duplicate and analyzed (100,000 cells per sample) using the FACSCalibur flow cytometer and CellQuestPro Software (BD Biosciences).

**Annexin V-FITC apoptosis staining.** Cells were plated at 70% confluence, serum-starved for 24 hours. The next day, cells were treated with 30 μM SU1498 or left untreated for 2 hours in low-serum medium (1% v/v FBS). Further, cells were either irradiated using X-ray generator (HF160 [Pan tak], Philips Medico; 150 kV, 15 mA, dose-rate 2.18 Gy/min) with 10 Gy of IR or left untreated, harvested 24 hours post-radiation treatment and stained with Annexin V-FITC conjugate according to manufacturer’s instructions (BD Biosciences). Cells that stained positive for Annexin V-FITC were considered as undergoing apoptosis. Experiments were performed 2 times in duplicate and analyzed (100,000 cells per sample) using FACSCalibur flow cytometer and CellQuestPro Software and BD Biosciences.

**Patients and tissues.** A series of 28 WHO grade I and II astrocytomas (LGA) and 35 WHO grade III and IV astrocytomas and glioblastomas, respectively (HGA) were investigated. Tumor samples were obtained from patients who underwent surgery at the Department of Neurosurgery, Faculty of Medicine and Dentistry, Palacky University, Olomouc, between 1989 and 2004. The grading and grading was reviewed independently by two pathologists according to WHO classification.

**Immunohistochemistry.** Indirect immunohistochemical detection of VEGF (antibody clone C-1, dilution 1:50, Santa Cruz Biotechnology, INC), Flt-1 (antibody clone C-19, dilution 1:50, Santa Cruz Biotechnology, INC), and KDR (Flk-1, antibody clone A-3, dilution 1:150, Santa Cruz Biotechnology, INC) on formalin (10%, pH 7.4) fixed, paraffin-embedded sections was performed according to standard procedures using a high temperature epitope retrieval technique in citrate buffer (10 mM, pH 6; microwave oven 20 min). Diaminobenzidine (DAB) was used as chromogen. Samples of normal skin fibroblast and vascular endothelial cells of normal mucosa of large intestine were used as markers of negativity of Flt-1/VEGF and KDR, respectively. Non-specific granulation tissue with newly formed vessels was used as a positive control for all antibodies studied (data not shown). Immunohistochemical staining was evaluated by a semi-quantitative method using the histoscore method, which is a multiplication of indexes (scores) obtained for the homogeneity (percentage of positive cells), and the intensity of staining. The latter was scored as weak (1), moderate (2) or strong (3) as described previously. The analysis and scoring was performed by two pathologists blinded to the clinical data.

**Statistical analysis.** Matched pair analysis was performed using T test and non-parametric Mann-Whitney test for the analysis of patients’ data. ANOVA (non-parametric Kruskal-Wallis test) and Student t-test were used in statistical evaluation of in vitro experiments (the values are given as the mean ± SD of three independent experiments). In all tests, a value of p ≤ 0.05 was considered as significant.

**Acknowledgements**

This work was supported by grants from the Czech Ministry of Education (MSM6198959216), Palacky University (IGUPO-1110131/39), the Czech Ministry of Health (IGAMZCRNR/8370-3), and the Lundbeck Foundation (R13-A1287).

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