Among the various adaptive responses to cell and tissue hypoxia is that of neoangiogenesis. This process occurs under physiological and pathophysiological conditions. It is becoming apparent that primary brain tumours of astrocytic lineage, especially glioblastoma multiforme, exhibit hypoxia-induced neoangiogenesis in vivo through induction of the endothelial cell-specific mitogen, vascular endothelial growth factor (VEGF) (Shweiki et al, 1992; Plate et al, 1992, 1994). A strong correlation was found between intense VEGF expression and severe hypoxia in situ in HT29 and EMT-6 spheroids (Waleh et al, 1995).

Recently we have observed variable presence of hypoxia adjacent to necrosis in some xenografted glioma lines and spheroids (Parliament et al, 1997; Franko et al, 1998). The results were interpreted in terms of regional variations in oxygen consumption, based on the concept of modulation of oxygen consumption in regions of oxygen and nutrient deprivation in solid tumours (Hochachka et al, 1996). We were interested in determining the pattern of VEGF expression in situ, particularly in the nutrient-deprived regions of viable cells adjoining necrosis, which may or may not be severely hypoxic in human glioma spheroids and xenografts. We recently showed that the M006XLo line has elevated expression of VEGF under aerobic conditions, with modest hypoxic induction compared to the M006X line (Allalunis-Turner et al, 1999). In this report we have examined the oxygen dependence of VEGF mRNA expression in the M006X line undergoing mild hypoxic stress (6% oxygen (O2)), and we have also examined VEGF expression in spheroids of both M006X and M006XLo lines and in xenograft tumours of the M059K, M006 and M010b lines. We also sought to independently validate the presence of radiobiologically hypoxic cells in situ in M006 tumours using the comet assay. Furthermore, this and our previous study demonstrate that levels of VEGF expression vary among sublines (M006, M006X and M006XLo) derived from a single human glioma specimen. © 2000 Cancer Research Campaign

Key words: hypoxia; glioma; vascular endothelial growth factor; misonidazole; flt-1

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Finally, we have performed preliminary experiments to assess the presence of tumour-cell VEGF receptor (Vaisman et al, 1990) expression which has previously been seen in melanoma and ovarian carcinoma lines (Boocock et al, 1995; Liu et al, 1995), suggesting autocrine/paracrine growth stimulation.

**MATERIALS AND METHODS**

**Cell lines, spheroids and xenografts**

Details of the origin and characterization of the glioma cell lines used in this study have been previously published (Parliament et al, 1997; Allalunis-Turner et al, 1991). The M0059K (passage...
Northern blotting

Gloma cells were grown as monolayers under standard incubator conditions (37°C, 5% carbon dioxide–95% air). To study the effect of changes in the gas-phase O2 concentration on VEGF expression, a gas exchange manifold system utilizing a series of leakproof aluminium chambers (Koch et al, 1979) was used to create atmospheres of varying proportions of oxygen in 5% carbon dioxide–95% N2. Total RNA was isolated from cells cultured for 24 h under aerobic or hypoxic conditions using the guanidinium–silica gel column method (Qiagen). Twenty micrograms of total RNA were electrophoresed in each lane. After transfer, the blots were hybridized with [32P random-labelled probes to VEGF mRNA were electrophoresed in each lane. After transfer, the blots were hybridized with [32P random-labelled probes to VEGF

Comet assay

Unanaesthetized NOD/SCID mice bearing M006 tumours were used for experiments when the tumours reached a size of 300–500 mg. Mice were restrained in lucite jigs and breathed room air. The tumours were irradiated with 15 Gy 250 kV X-rays at a dose rate of 3.3 Gy min⁻¹. Tumours were excised within 30 s at the end of radiation exposure and disaggregated in ice-cold phosphate-buffered saline (PBS). Cell suspensions were mixed with agarose (final concentration 0.75%), lysed with an alkaline high salt lysis solution, rinsed in alkali, and exposed to 0.6 volts cm⁻¹ in an electrophoresis chamber as previously described (Olive et al, 1997). DNA was stained with propidium iodide and individual cell ‘comets’ were viewed using a Zeiss epifluorescence microscope attached to an intensified solid state charge device camera and image analysis system (Olive et al, 1997). The hypoxic fraction was estimated by iterative fitting of histograms of ‘tail moment’ (product of the percentage of DNA in the comet tail multiplied by the distance between the means of the head and tail distributions) with two normal distributions, representing the aerobic and hypoxic populations as described previously (Olive et al, 1997).

Histologic preparation

After growth to diameters of 800–1000 μm spheroids of the M006 and M006Lo lines were labelled under aerobic conditions with 50 μM 3H-misonidazole for 3 h as described previously (Franko et al, 1998). The specific activity of 3H-misonidazole was 1.045 × 10¹⁰ Bq mol⁻¹. Tumours were labelled with 3H-misonidazole with three injections (of the same specific activity drug as noted above) at 1-h intervals into mice at a dose equivalent to a whole body concentration of 50 μm, as described previously (Parliament et al, 1997). The spheroids and tumours were either fixed in 10% buffered formalin at 4°C overnight, then at room temperature for at least 3 days, or fixed in methanol–chloroform–glacial acetic acid (6:3:1 by volume; methanol-Carnoy’s) at 4°C for 30 min, then overnight at room temperature. The material was then embedded in wax, cut at 5 μm and mounted on poly-l-lysine-coated slides.

VEGF ISH

The human VEGF 204 bp probe described above was labelled with biotin using a T7 RNA polymerase using biotin-14-CTP (Gibco-BRL) according to manufacturer’s instructions. Spheroid and xenograft (M006X) sections, which had been fixed with formalin, were dewaxed and blocked with levamisole to block detection of endogenous biotin. In situ hybridization (ISH) detection utilized streptavidin linked to NBT (Oligo Colour Kit, Amersham Life Sciences). Semi-quantitative assessment (after Plate et al, 1994) of blue colour reaction was undertaken and compared to the H-misonidazole autoradiography on serial sections (see below). The blue colour intensity was graded by one observer as: (−) = absent; (+) = light; (++) = moderate; (+++) = heavy; and (+++++) = very heavy.

VEGF immunohistochemistry

Deparaffined slides underwent unmasking with hot citric acid (2.1 g L⁻¹ at pH 7.3 for 30 min at 70°C), then were rinsed with PBS containing 10% goat serum. Slides were then incubated with the primary rabbit anti-human VEGF antibody, 1:150 (Santa Cruz A-20 raised to amino acid residues 1–20, recognizing VEGF 121, 165, 189 splice variants). The primary antibody was detected using a Fast Red detection kit (BioGenex). Slides were lightly

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counterstained with haematoxylin, in order to emphasize positive immunohistochemistry (IHC) staining. On control slides, the antibody was neutralized by incubation with a tenfold excess of control VEGF peptide (Santa Cruz P-20) prior to the anti-VEGF step. Additional controls in preliminary experiments used rabbit IgG in place of the primary antibody.

3H-misonidazole autoradiography

Sections adjacent to those used for ISH and IHC were dewaxed, dipped in NTB-2 emulsion and exposed for 4–6 weeks. After developing and fixing, the sections were stained through the emulsion with haematoxylin and eosin. Grain density was counted regionally at 1000× using a 10×10 μm microscope grid.

VEGF receptor (flt-1) IHC

M006XLo slides were prepared as for VEGF IHC. These slides were then incubated with a polyclonal rabbit anti-human flt-1 antibody (Santa Cruz). The primary antibody was detected with the Fast Red detection kit (BioGenex).

RESULTS

Northern blotting

Northern blot analysis of VEGF mRNA expression in M006X cells incubated at various O2 concentrations is shown in Figure 1. VEGF expression increased markedly with exposure to increasing degrees of hypoxia. Levels of β-actin mRNA were comparable at 18, 6 and 2% O2, and were slightly decreased under anoxia. Normalizing the (VEGF/actin) ratio in room air to unity, the degree of VEGF induction observed under 6% O2, 2% O2, and anoxia (100% N2) was as follows: 3.7-, 9.5-, and 29.8-fold respectively.

Hypoxic fraction of M006 xenografts measured using the comet assay

In 12 tumours from air-breathing mice, the mean hypoxic fraction was 8.9 ± 7.0% (range 0–21%). These results are summarized in Table 1.

| Tumour number | Treatment | Proportion of hypoxic cells (curve fitting) |
|---------------|-----------|-------------------------------------------|
| 1             | Air-breathing | 0.10                                      |
| 2             | Air-breathing | 0.034                                     |
| 3             | Air-breathing | 0.01                                      |
| 4             | Air-breathing | 0                                         |
| 5             | Air-breathing | 0.137                                     |
| 6             | Air-breathing | 0.042                                     |
| 7             | Air-breathing | 0.071                                     |
| 8             | Air-breathing | 0.21                                      |
| 9             | Air-breathing | 0.107                                     |
| 10            | Air-breathing | 0.075                                     |
| 11            | Air-breathing | 0.067                                     |
| 12            | Air-breathing | 0.21                                      |
| 13            | Clamped      | 0.81                                      |
| Mean ± SD     | Air-breathing | 0.089 ± 0.07                              |

Relationship of VEGF mRNA expression to hypoxia marker binding in glioma spheroids and xenografts

Aerobic M006XLo spheroids (Figure 2) and M006X spheroids showed strong staining for the hybridized VEGF probe across the entire viable rim. Semiquantitative assessment of ISH staining was made and compared with the intensity of 3H-misonidazole labelling on contiguous 5-μm sections (Table 2). Heterogeneity of 3H-misonidazole labelling adjacent to necrosis was seen from spheroid to spheroid within the same experimental flask as reported previously (Franko et al, 1998). Some spheroids displayed heavy labelling in the inner five cell layers adjacent to necrosis, indicating a substantial gradient of [O2], and severe hypoxia adjacent to necrosis, while others were more lightly labelled indicating aerobic levels of oxygen throughout the spheroids (Franko et al, 1998). There was no apparent relationship between VEGF expression and hypoxic marker binding, although in one of ten spheroids, elevated 3H-misonidazole binding and VEGF ISH staining were seen to co-localize to the innermost cells. A similar relationship between VEGF and hypoxia marker binding existed in M006X spheroids, which show lower levels of aerobic expression of VEGF in vitro than the M006XLo line (data not shown).
VEGF ISH in M006X tumours showed uniform staining in all tumours examined except one, where there was increased staining adjacent to approximately 20% of necrotic regions (Figure 3A, 43; Figure 3B 103). Five tumours were assessed, with identical findings. Similar findings were also noted for M006XLo (two tumours), M010bX (one tumour) and M0059KX (two tumours).

VEGF IHC

Immunoreactivity to the anti-VEGF antibody was superior for tumours and spheroids fixed with methanol-Carnoy’s compared to those fixed with formalin. The staining was more intense and the concentration difference between non-specific staining with control IgG and acceptable staining with the antibody was at least threefold better for methanol-Carnoy’s. Control sections in which the primary anti-VEGF antibody was neutralized by a large excess of control VEGF peptide were uniformly negative. In all tumours assessed of the M059KX, M006X, M006XLo and M010bX lines (four tumours from each), and in M006XLo spheroids, immunodetection of VEGF protein showed similar staining across all regions of healthy cells up to the border of necrosis (Figure 4A). In preliminary experiments which used a wide range of dilution of the primary antibody, the lightest staining visible appeared to be similarly uniform, with no suggestion of preferential staining of cells adjacent to necrosis. On contiguous sections, simultaneous assessment of hypoxia marker binding indicated marked heterogeneity, consistent with previous studies (Parliament et al, 1997). These tumour regions included cells exhibiting a full range of 3H-misonidazole grain densities (from fully aerobic to hypoxic). A similar result was apparent using formalin-fixed tissue (data not shown). These results indicate an absence of a correlation in situ between [O2] and VEGF protein expression.

VEGFR (flt-1) IHC

Using a similar protocol to detect VEGFR (flt-1) in M006XLo cells, we observed strong immunostaining for flt-1 in all tumour cells of this preparation (Figure 5).

Table 2 3H-miso autoradiography vs VEGF ISH in M006XLo glioma spheroids

| Spheroid number | 3H-miso grain densitya | VEGF ISH intensityb |
|----------------|------------------------|---------------------|
|                | Outer layers | Inner layers | Outer layers | Inner layers |
| 1              | +          | +           | +          | +           |
| 2              | +          | +           | +          | +           |
| 3              | +          | +           | ++         | +++         |
| 6              | +          | +           | +++        | +++         |
| 10             | +          | +           | ++++       | +++         |
| 4              | +          | ++          | ++         | ++          |
| 5              | +          | ++          | ++         | ++          |
| 8              | +          | +++         | ++         | +++         |
| 9              | +          | +++         | +++        | +++         |

aEach extra (+) indicates an increase in grain density by a factor of approximately 8. bIntensity was graded by one observer as (−) = absent; (+) = light; (++) = moderate; (+++) = heavy; (++++) = very heavy.
The understanding of neoangiogenesis in glioblastoma multiforme has advanced significantly in the last 4–5 years. Indeed, the presence of necrosis and vascular proliferation distinguishes glioblastoma multiforme from most other malignant brain tumours including astrocytomas (Germano et al, 1989). Previous investigators (Plate et al, 1992; Shweiki et al, 1992) noted marked up-regulation of VEGF expression in pallisading cells adjacent to necrosis and also in other small anaplastic cells at some distance from necrosis. It is now certain that in addition to hypoxic stress (Shweiki et al, 1992; Damert et al, 1997), VEGF may be induced by CoCl₂ (Goldberg and Schneider, 1994); nitric oxide (Chin et al, 1997); tumour necrosis factor-α (TNF-α) (Ryuto et al, 1996); glucose deprivation (Stein et al, 1995); platelet derived growth factor-BB; transforming growth factor-β (TGF-β) (Brogi et al, 1994; Frank et al, 1995); and interleukin-1β (Li et al, 1995). The presence of multiple metabolic and growth factor modulators of this endothelial mitogen imply a complex in vivo regulation. In contrast to the finding of co-localization of VEGF expression and hypoxia using the marker EF5 in HT29 and EMT-6 spheroids (Waleh et al, 1995), we found discordant results in several human glioma lines grown as multicellular spheroids and xenografts. Both VEGF ISH and IHC revealed virtually ubiquitous staining regardless of the proximity to nutrient capillaries or necrosis (Figures 3 and 4A, B). Only in a single xenograft tumour (M006X) was there a suggestion of increased VEGF mRNA expression adjacent to necrosis. Low power photomicrographs have been used to emphasize general uniformity of both VEGF ISH and IHC staining, with the ISH results confirmed by IHC.

Specific riboprobes that allow for the identification of the 121, 165, 189 and 206 amino acid isoforms of VEGF have been developed. The VEGF probe used in these studies, however, does not distinguish between the various VEGF mRNA species. In cardiac myocytes (Levy et al, 1995a), no significant changes in relative isoform amounts were observed in response to hypoxic stress. Given that the induction of VEGF mRNA species, using the probe in our Northern analysis, results in the classically described picture of VEGF mRNA induction with hypoxia, it is unlikely that the absence of variations in expression of VEGF in situ is simply related to changes in the proportions of isoforms without a change in total transcript number.

The findings in the current models differ from those originally described (Shweiki et al, 1992) in patient-derived primary biopsies. Every effort has been made to use glioma cell lines which are preserved in an early passage stage after explantation (Parliment et al, 1997). However, in situ oxygenation was not assessed in the original study (Shweiki et al, 1992) of VEGF overexpression; hypoxia was merely presumed to exist adjacent to necrosis. To our knowledge, our data represent the first examination of the relationship between VEGF expression and hypoxia in a solid glioma tumour model to date. A similar correlative study would be possible in patients, but would require in situ hypoxia marker labelling prior to biopsy (Urtasun et al, 1986). The hypoxic fraction values obtained with the M006 xenografts for the comet assay are close to those of the C3H murine mammary tumour under air-breathing conditions (Olive et al, 1997) and are consistent with our 3H-misonidazole data suggesting that most but not all M006 xenografts contain substantial radiobiological hypoxia.

The oxygen dependence of misonidazole binding is slightly different from the hypoxic marker EF5 (Koch et al, 1995). EF5 shows less inhibition of binding at moderate oxygen tensions, whereas misonidazole binding is inhibited by oxygen in a somewhat more complex fashion down to levels of hypoxia which would be considered significant from the point of view of induction of radiation resistance (Chapman, 1984). In spheroids we have also seen heterogeneous binding patterns of pimonidazole (Kennedy et al, 1997), adjacent to the necrotic centres in a fashion identical to that seen with misonidazole (AI Franko, unpublished data). Therefore, the findings in this study do not appear to be an artifact of one particular hypoxia marker. Lack of correlation between hypoxia marker binding and VEGF expression in xenograft tumours is somewhat complex in that intratumoural drug distribution may be suboptimal because of the aberrant tumoural vasculature. However, glioma spheroids (Franko et al, 1998), in which nitroimidazole drug penetration is not impaired, also reveal substantial variation in hypoxia marker binding in the viable cells adjacent to necrosis, a pattern similar to that seen in xenograft tumours.

From the Northern analysis it is apparent that severe, pathophysiological levels of hypoxia are not necessary for the up-regulation of VEGF messenger RNA and protein expression; indeed substantial upregulation is evident at modest levels of O₂, which would be similar to the physiological hypoxic conditions noted in many mammalian organ systems. Our results are in agreement with those of Leith and Michelson (1995) who showed that modest hypoxia (i.e. 10% O₂) is also sufficient to cause some induction of VEGF expression in the clone A human colon cancer cell line. VEGF production is known to be mediated by both transcriptional and post-transcriptional mechanisms (Levy et al, 1995b, 1997). It is interesting to note that transcriptional regulation of VEGF expression, through binding of the hypoxia-inducible factor-1 (HIF-1) to a 28 bp site in the VEGF 5′ promoter (Wang et al, 1995; Jiang et al, 1996) also occurs at physiological levels of hypoxia with half maximal response between 1.5 and 2% O₂ (Jiang et al, 1996). Post-transcriptional regulation of VEGF mRNA levels appears to be due to reversible stabilization of VEGF mRNA by hypoxia (Shima et al, 1995; Stein et al, 1995; White et al, 1995; Levy et al, 1996). However, constitutive stabilization of VEGF can occur through inactivation of the von Hippel–Lindau tumour suppressor gene (Wizigmann-V oos et al, 1995; Gnarra et al, 1996; Iliopoulos et al, 1996; Levy et al, 1996). We are currently investigating the possibility that such post-transcriptional stabilization is involved in the constitutive expression of VEGF by the lines M006XLo and M059K.
The Northern blot data for M006X cells in vitro demonstrate that VEGF expression is unregulated by 3.5-fold following a reduction in pO₂ from 18% to the more physiologically relevant level of 6%. Furthermore, we have recently demonstrated IHC detection of increased expression of VEGF protein in cultured hypoxic (0.6 and 2% O₂) M006X cells (Allalunis-Turner et al., 1999). In vitro, the percentage of M006X cell staining positively by IHC for VEGF increased from 17% under fully oxygenated conditions to 97% (under 2% O₂). These data indicate that much of the VEGF mRNA induction detected by Northern blotting is a result of increase in the proportion of cells expressing VEGF rather than a uniform increase in VEGF mRNA in most cells. A change in staining pattern of this type would have been clearly visible in the IHC of the spheroids and tumours. These data are also compatible with the detection of VEGF mRNA and protein in presumably well oxygenated tumour cells adjacent to blood vessels in vivo. However, the Northern data do not provide an explanation for the lack of an increase in VEGF expression with increasing distance from blood vessels, and particularly the absence in almost all tumours of elevated expression in severely hypoxic cells adjacent to necrosis. An increase in expression by at least fourfold (possibly eightfold) in the latter areas was predicted by the Northern data, and clearly this was not observed. The possible biological reasons for the disparity between our in vitro and in vivo results require further investigation.

Up to 50% of the necrotic areas in M006 and M006XLo spheroids and M059K and M006 xenografts were reported to be well oxygenated (Parliament et al., 1997; Franko et al., 1998), and those observations are confirmed by the data reported here. The cause of cell death in these regions has not been identified, although we have postulated that it is related to glucose insufficiency (Franko et al., 1998), which is known to up-regulate VEGF expression (Stein et al., 1995). The absence of up-regulation of VEGF expression in cells adjacent to both aerobic and hypoxic necrosis suggests that its regulation in glialoma cells is remarkably resistant to various types of microenvironmental stress.

Recently, a quantitative immunohistochemical comparison of hypoxia and VEGF protein expression in squamous cell carcinomas of the uterine cervix and head and neck has been reported (Raleigh et al., 1998). In this study in which patients received the hypoxia marker pimonidazole prior to biopsy, no correlation was seen between hypoxia and VEGF protein expression on quantitative image analysis of histologic sections. The authors considered the possibility that the lack of a correlation was a consequence of the fact that the half-maximal inhibition of 2-nitroimidazole binding by oxygen (0.4% gas-phase concentration) occurs at a lower [O₂] than half-maximal VEGF up-regulation (0.8–2.2% gas-phase concentration) in mammalian cells. While this means that pimonidazole labelling would not identify all cells capable of inducing VEGF expression via a hypoxia-driven mechanism, the authors argued that substantial gradients of VEGF expression should have been observed in the cells separating blood vessels and severely hypoxic cells. These were not observed, which lends support to our observation that VEGF expression is independent of hypoxia in these glioma models.

While one may speculate that cell lines overexpressing VEGF when grown as tumours in vivo may achieve a growth advantage because of a ‘pro-angiogenic’ phenotype, we have considered another possible hypothesis. Reports of expression of VEGF receptors KDR (Boocock et al., 1995; Liu et al., 1995) and flt-1 (Boocock et al., 1995) have suggested a possible autocrine/paracrine growth stimulation by VEGF in certain melanoma and ovarian carcinoma lines. In view of this possibility, VEGFR-1 (flt-1) expression in the M006XLo xenograft was analysed. Strong, widespread expression of VEGFR-1 was detected in tumour cells. Previous studies utilizing biopsies from patients with gliomas have reported only tumour endothelial cell expression of VEGF receptors (Plate et al., 1994). Confirmatory studies are required to establish whether autocrine stimulation of growth is occurring in these lines.

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