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Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: Implications for tumor angiogenesis

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ABSTRACT **Perfusion insufficiency, and the resultant hypoxia, often induces a compensatory neovascularization to satisfy the needs of the tissue. We have used multicellular tumor spheroids, simulating avascular microenvironments within a clonal population of glioma tumor cells, in conjunction with *in situ* analysis of gene expression, to study stress inducibility of candidate angiogenic factors. We show that expression of vascular endothelial growth factor (VEGF) is upregulated in chronically hypoxic niches (inner layers) of the spheroid and that expression is reversed when hypoxia is relieved by hyperoxygenation. Acute glucose deprivation—another consequence of vascular insufficiency—also activates VEGF expression. Notably, glioma cells in two distinct regions of the spheroid upregulated VEGF expression in response to hypoxia and to glucose starvation. Experiments carried out in cell monolayers established that VEGF is independently induced by these two deficiencies. Upon implantation in nude mice, spheroids were efficiently neovascularized. Concomitant with invasion of blood vessels and restoration of normoxia to the spheroid core, VEGF expression was gradually downregulated to a constitutive low level of expression, representing the output of nonstressed glioma cells. These findings show that stress-induced VEGF activity may compound angiogenic activities generated through the tumor "angiogenic switch" and suggest that stress-induced VEGF should be taken into account in any attempt to target tumor angiogenesis.**

Growth of solid tumors is thought to be dependent on neovascularization (1). Tumor progression is associated with multiple genetic changes, including changes leading to increased production of angiogenic factor(s). For example, the progression from a benign, avascular astrocytoma to a malignant, highly vascularized glioblastoma multiforme is thought to include such an "angiogenic switch." Deficient vascularization of malignant tumors results in development of different microenvironments within the tumor mass that may in turn affect gene expression. Certain cellular responses to a deficient oxygenation and nutrient supply are of an adaptive nature. Notably, hypoxia is thought to elicit a compensatory feedback response of neovascularization. It is reasonable, therefore, that stress-induced angiogenic factors may play an important role in recruitment of new blood vessels into undervascularized tumors.

To uncover stress-induced angiogenic factors, we have previously analyzed tumor biopsies representing imbalanced vascularity with respect to spatial pattern of expression of candidate genes. Using this procedure, we have shown that vascular endothelial growth factor (VEGF) is specifically induced in a subset of glioblastoma cells distinguished by their immediate proximity to necrotic foci (presumably hypoxic microregions) (2, 24). VEGF is a secreted, 46-kDa dimeric protein active as an angiogenic growth factor, as an endothelial cell-specific mitogen, and as a vascular permeability factor (3–8).

The complex multicellular organization of primary tumors makes it difficult, however, to distinguish stress-induced expression from expression associated with altered differentiated phenotypes of tumor subsets. It is also difficult to distinguish hypoxia-induced expression from expression induced by other deficiencies or from indirect effects of necrosis factors. To overcome these shortcomings, we have resorted to analysis of three-dimensional multicell spheroids. Spheroids grown from established cell lines represent a clonal cell population in which gradients of oxygen, glucose, and other nutrients create a continuum of different microenvironments (refs. 9 and 10 and references therein). The spheroid system thus simulates microregions that develop in undervascularized tumors at various distances from the nearest capillaries. Furthermore, the shapes of oxygen and glucose concentration gradients can be experimentally altered and effects on gene expression can be determined under conditions that simulate acute or chronic deficiencies.

Using spheroids grown in different conditions, we establish that VEGF expression is hypoxia inducible. Furthermore, we uncover a second, independent trigger for VEGF induction—namely, glucose deficiency. We also used implanted, neovascularizing spheroids to evaluate the *in vivo* relevance of stress-induced VEGF activity.

MATERIALS AND METHODS

Cell Culture and Spheroid Preparation. C6 cells, a clonal glial cell line derived from a rat glial tumor (11), were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and antibiotics. Aggregation of cells into small spheroids of 400–800 μm was initiated by incubating cells from confluent cultures in nonadherent bacteriological dishes for 48 h. Emerging spheroids were then transferred into spinner flasks, and, after an additional 10 days of growth, spheroids were sorted to uniform size by sedimentation through a 10-ml pipette. Mean spheroid diameter at 23 days of culture was 313 μm (SD = 30 μm; n = 50). Approximately 300 spheroids were cultured in 500 ml of medium. Growth continued for ≈6 weeks with a spinning rate of 80 rpm and a change of medium every other day. A mixture of 95% air/5% CO_2 was blown over the medium every 48 h for 5 min to ensure adequate oxygenation and pH buffering. Spheroid growth followed Gompertz kinetics (A = 0.315 day^{-1}, B = 0.022 day^{-1}).

Hyperoxygenation and Hypoxia Induction. Hyperoxygenation of spheroids was achieved by saturating the medium with 95% oxygen/5% CO_2 as follows: serum-free medium was bubbled with the gas mixture for 4 h. After addition of serum and spheroids, the oxygen/CO_2 mixture was blown over the

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Abbreviation: VEGF, vascular endothelial growth factor.

medium for an additional 20 min, after which the flask was sealed. Spheroids were withdrawn into a fixative after 16 h of incubation.

To achieve hypoxia in C6 monolayers, near confluent cultures were incubated in a GasPak Plus anaerobic culture chamber (BBL Microbiology Systems), using hydrogen and a palladium catalyst to remove all traces of oxygen. Exposure to hypoxic conditions was for 18 h.

Isolation and Blot Analysis of RNA. Total RNA was prepared by the guanidine thiocyanate extraction method and was purified by centrifugation through CsCl solution (12). RNA was denatured in glyoxal and electrophoresed through a 1.0% agarose gel. RNAs were transferred onto a nylon-based membrane (GeneScreen*Plus*; NEN) by the capillary blot procedure and were hybridized with VEGF cDNA labeled with ^{32}P by randomly primed DNA synthesis.

***In Situ* Hybridization.** Free spheroids or resected implanted spheroids were fixed in 4% paraformaldehyde. Frozen specimens were sectioned (10 μm thick), processed, and hybridized *in situ* as described (13). Autoradiographic exposure was for 5–9 days. As a VEGF-specific probe, we used a 1.8-kB cDNA fragment containing approximately the 3′ two-thirds of the coding region as well as the entire 3′ untranslated region of mouse VEGF. cDNA was subcloned onto the polylinker of a PBS vector (Stratagene) and was linearized by digestion with the appropriate restriction endonuclease to allow synthesis of a ^{35}S-labeled complementary RNA in either the antisense or sense orientation (using T3 or T7 RNA polymerase, respectively). RNA probes were fragmented by mild alkaline treatment before use for *in situ* hybridization.

Visualization of Proliferating Cells by BrdUrd Staining. Spheroids were incubated for 4 h in BrdUrd (5-bromo-2′-deoxyuridine)-containing medium, transferred to 0.5 M sucrose in PBS, embedded, and sectioned as described (13). Sections were fixed in 100% ethanol (10 min) and reacted with anti-BrdUrd antibodies, using reagents and procedures from a cell proliferation kit (Amersham; RPN20).

RESULTS

Upregulated VEGF Expression in Inner (Hypoxic) Layers of Glioma Spheroids Is Reversed by High Oxygen. Spheroids were prepared from an established cell line of a rat glioma tumor (line C6) as described. After reaching 0.6–0.8 mm in diameter, spheroids were fixed and processed for *in situ* analyses. *In situ* analysis of cell proliferation was carried out by visualizing cells that have incorporated BrdUrd into newly replicated DNA. Results showed that cell proliferation was mostly confined to the outermost three to five cell layers, while the remainder of viable tumor cells (≈70%) were quiescent. The spheroid core was clearly necrotic (Fig. 1).

To determine the spatial distribution of VEGF-expressing cells, thin sections were hybridized with a VEGF-specific probe. Fig. 1*A* shows that cells of the inner core expressed significantly higher levels of VEGF mRNA than cells in the outer layers. The simplest interpretation of this result is that differences in expression result from microenvironmental differences and, specifically, that VEGF expression was upregulated in stressed cells that did not receive sufficient oxygen or nutrients by diffusion.

To determine whether improved delivery of oxygen to the inner layers will result in downregulation of VEGF expression, spheroids from the same batch shown in Fig. 1*A* were transferred to a medium saturated with 95% oxygen and were incubated for an additional 16 h prior to *in situ* analysis. As shown in Fig. 1*B*, VEGF expression in the inner layers was downregulated to the same low level of expression detected in peripheral cell layers. The fact that hyperoxygenation abolished excessive expression of VEGF ruled out the possibility that augmented expression was due to a particular differentiated phenotype emerging in the course of spheroid growth. In addition, this result ruled out the possibility that VEGF expression was stimulated by a factor emanating from nearby necrosis.

VEGF Expression Is Upregulated in Glucose-Deprived Cells. Inadequate perfusion will eventually lead to a deficiency in tissue glucose. Glucose deprivation in turn is known to induce expression of a number of genes (14–16). Rationalizing that glucose deficiency may also serve as a trigger for a feedback angiogenic response, we determined whether VEGF expression is upregulated by low glucose. Spheroids were acutely exposed to a low glucose-containing medium (0.1 g/liter), and spatial distribution of VEGF-expressing cells was determined by *in situ* hybridization. As shown in Fig. 2*A*, exposure to low glucose resulted in the activation of VEGF expression, specifically in the peripheral cell layers. Noteworthy, this pattern of expression is different and, in fact, reciprocal to the pattern of expression detected in spheroids grown in normal glucose (compare with Fig. 1*A*). In particular, VEGF was not upregulated in the hypoxic core of the spheroid, suggesting that glucose-starved cells are unable to induce VEGF expression in response to hypoxia (see below).

While certain stress proteins are known to be induced by oxygen deficiency (ORPs) (16–18), others may be primarily regulated by glucose deficiency (GRPs), and some may be regulated by both. We wished to determine how these two insults interact in regulating VEGF expression in the context of an avascular, three-dimensional tumor mass. To produce different combinations of oxygen and glucose in expanding spheroids, we analyzed spheroids that were transferred from a normal (4.5 g/liter) to a low glucose-containing medium (0.1 g/liter) and were retrieved for analysis relatively shortly (6 h) thereafter. We assumed that increasing diffusion distances, in conjunction with differential rates of glucose consumption by proliferating versus quiescent cells and by normoxic versus hypoxic cells (18), will lead to a wide range of glucose/oxygen ratios. As shown in Fig. 2 *B* and *C*, under these conditions VEGF mRNA expression was induced in two distinct microenvironments: in peripheral layers, presumably representing normoxic, glucose-deficient cells (that might also be proliferating) and in a subset of inner cells that are hypoxic and quiescent. Note that expression was mostly detected in relatively narrow "bands" of cells, separated by several layers of nonexpressing cells (Fig. 2*C*). Thus, different combinations of suboptimal glucose and oxygen, which may develop in noncontiguous regions of the tumor, induce expression of the angiogenic factor.

Induction of VEGF Expression by Hypoxia and by Glucose Deficiency Are Mutually Exclusive. To better define optimal combinations of glucose and oxygen required for VEGF induction, we regressed to a monolayer system where glioma cells of the same cell line were grown in medium containing different glucose concentrations and were exposed to different oxygen tensions. As shown in Fig. 3, VEGF was maximally induced under two sets of experimental conditions: glucose starvation in conjunction with normoxia and hypoxia in conjunction with normal and high glucose concentrations. Importantly, doubly stressed cells (i.e., cells deprived of both glucose and oxygen) were unable to upregulate VEGF expression.

VEGF Expression in Avascular Regions of the Tumor Is Downregulated upon Neovascularization *in Vivo*. Relief of hypoxia from the spheroid core *in vitro* resulted in decreased expression of VEGF (Fig. 1). We wished to determine whether restoration of normoxia in a physiological manner—namely, through natural neovascularization—will have the same effect. To this end, preformed spheroids, 0.6–0.8 mm in diameter, were injected intraperitoneally into nude mice. Most spheroids were readily implanted on the peritoneum or on other internal organs and underwent efficient neovascularization (Fig. 4*A*). Spheroids were resected at daily intervals during the 1st week

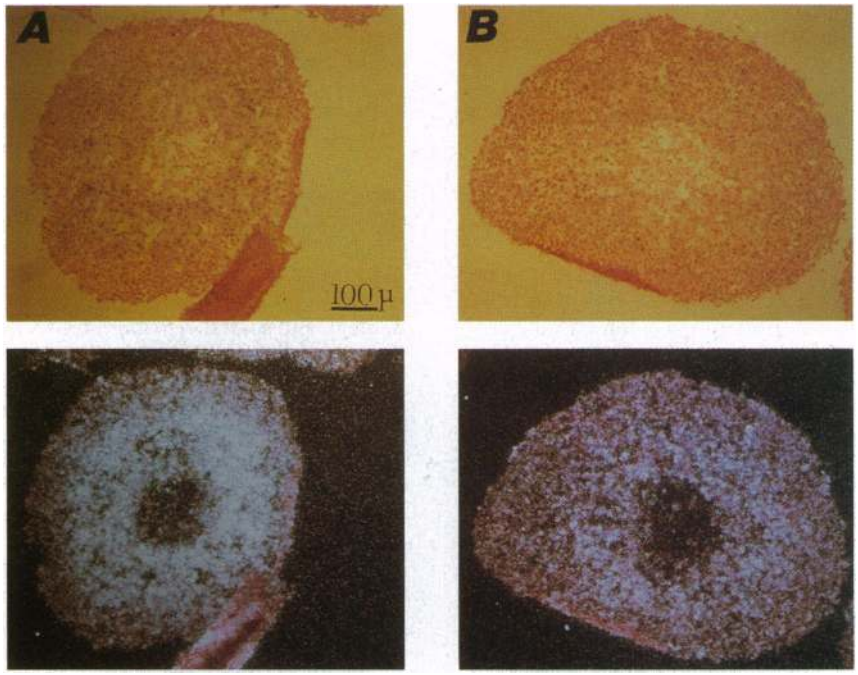

FIG. 1. *In situ* hybridization of glioma spheroids with a VEGF-specific probe. (*A*) Spheroid cultured in a medium saturated with a mixture of 95% air/5% CO_2. (*B*) Hyperoxygenated spheroid exposed for an additional 16 h to a mixture of 95% oxygen/5% CO_2. Thin sections were hybridized with a VEGF-specific probe and counterstained with hematoxylin and eosin. To better visualize autoradiographic grains, slides were photographed under bright-field (*Upper*) and dark-field (*Lower*) illuminations.

postinoculation, sectioned, and processed for *in situ* analysis. This procedure allowed us to compare patterns of expression in avascular, partially vascularized, and fully vascularized spheroids. In partially vascularized spheroids necrotic cells were replaced with viable cells only in areas proximal to the implantation site, while the distal part remained necrotic. As shown in Fig. 4*B*, the territory of intense VEGF expression has regressed to the distal part of the spheroid, adjacent to the remaining necrotic region. Upon further neovascularization, and adequate perfusion of the entire spheroid, VEGF expression was downregulated to a low, spatially uniform level of expression (Fig. 4*C*). Note that a low level of VEGF expression persisted in the fully vascularized spheroid (compare autoradiographic grain density within the spheroid to the background found in host tissues). This level of expression represents the inherent capacity of this highly angiogenic tumor to express

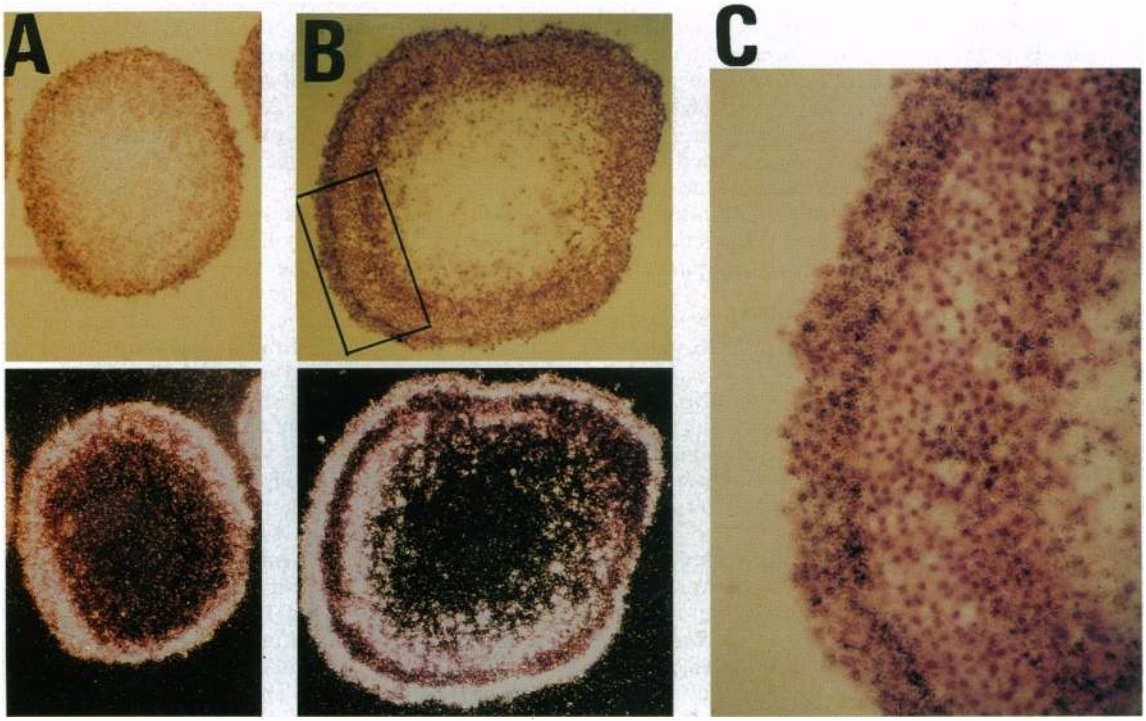

FIG. 2. Expression of VEGF in spheroids grown in different glucose concentrations. (*A*) Spheroids cultured under standard conditions (glucose, 4.5 g/liter) were grown in a low glucose-containing medium (0.1 g/liter) for 24 h prior to analysis. (*B*) Spheroids were transferred to glucose (0.1 g/liter) 6 h prior to fixation. (*C*) High-power view of the area boxed in *B*.

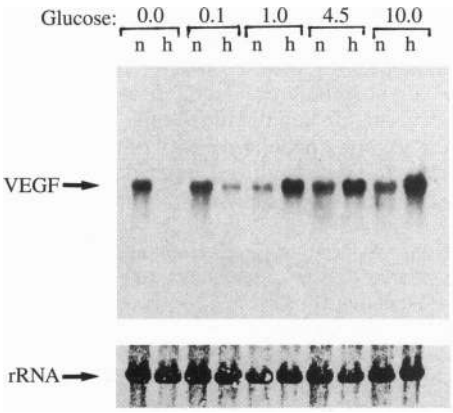

FIG. 3. Effects of glucose and oxygen on VEGF mRNA expression by C6 cells grown as monolayers. C6 monolayers were exposed for 8 h to a medium containing the indicated concentrations (percent) of glucose under either normoxic (n) or hypoxic (h) conditions. VEGF mRNA was detected by RNA blot hybridization. For standardization, blots were stained with methylene blue, and the 18S rRNA band is shown.

VEGF constitutively. The relative contribution of stress-induced VEGF can be appreciated from comparison with spheroids that were injected to the same animal but failed to implant and thus remained avascular (Fig. 4*C*).

DISCUSSION

The study addresses a fundamental issue in angiogenesis research. Namely, how do underperfused tissues "sense" a deficiency in oxygen and other nutrients and elicit a feedback neovascularization response to satisfy the demands of the tissue. This issue has important medical implications, including, for example, ischemia-induced development of collateral blood vessels and pathological intraocular neovascularization induced by retinal ischemia. Results presented here highlight the role of VEGF as a potential mediator of stress-induced neovascularization. It should be pointed out, however, that this study is concerned only with VEGF regulation. The angiogenic consequences of VEGF production and in particular inhibition of tumor angiogenesis by inhibiting VEGF action have been shown (19, 20).

Spheroids grown from an established glioma cell line were used to re-create different microenvironments within a population of otherwise identical cells. This system is thought to simulate a situation found in underperfused solid tumors where different microenvironments develop as a result of increasing distances from nourishing capillaries (10). The spheroid system circumvents a major problem of tumor analysis *in vivo*, which is the difficulty of distinguishing between local environmental effects and differences due to cellular heterogeneity. The usefulness of spheroids to uncover stress-regulated genes is enhanced by the fact that it is possible to

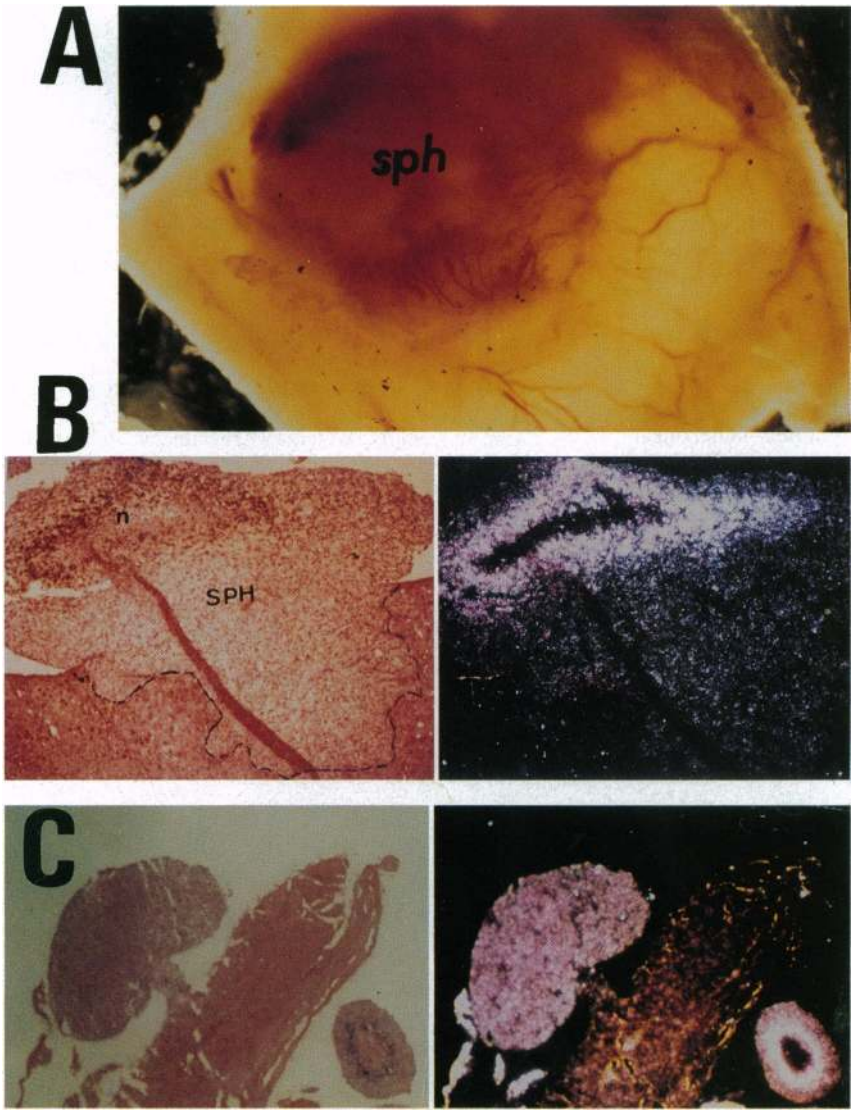

FIG. 4. VEGF mRNA expression in implanted, neovascularized spheroids. C6 spheroids were injected i.p. to nude mice. (*A*) Extensive neovascularization of a spheroid (sph) implanted on the peritoneum. (*B*) *In situ* hybridization of a spheroid resected 3 days after implantation (broken line shows spheroid boundary). Note that hybridization is maximal around the proximal necrotic region (n). (*C*) Fully vascularized spheroid resected 7 days postimplantation. Note relative levels of VEGF mRNA in the vascularized spheroid, host tissue, and the adjacent avascular spheroids that were injected to the same animal but failed to implant.

experimentally create a wide range of concentration gradients of oxygen and essential nutrients and is particularly useful in complex situations where optimal combinations of more than one factor are required for stress inducibility. Unless complemented by direct measurements of oxygen and glucose gradients and because of the interdependence of oxygen and glucose consumption, it is difficult to interpret the results with respect to the exact trigger of VEGF induction. Nevertheless, it is clear from Fig. 2 *B* and *C* that an acute glucose deficiency, superimposed on an oxygen gradient, created two distinct microenvironments conducive for VEGF induction. Similar situations may develop *in vivo* due to transient closure of vessels leading to intermittent perfusion of certain regions of the tumor (21). Confirmation of the notion that VEGF can be induced by either glucose or oxygen deficiency comes from the monolayer experiments (Fig. 3).

This study substantiated our previous findings that VEGF expression is hypoxia inducible (2). Importantly, the finding that VEGF expression in the spheroid core is abolished by elevating the concentration of external oxygen (Fig. 1) proved that the trigger for VEGF induction was hypoxia *per se* and unequivocally ruled out genetic heterogeneity as the source of differences in expression.

The study uncovered a second physiological stress that activates VEGF—namely, glucose deprivation. Noteworthy, basic fibroblast growth factor—another important angiogenic factor—was not regulated in a similar manner (data not shown). This finding adds VEGF to a growing list of glucose-regulated proteins that also includes GLUT-1 and the stress proteins GRP78 and GRP94 (22, 23). However, VEGF is the first angiogenic factor shown to be induced by glucose starvation. Importantly, we find that when cells grown as a monolayer are deprived of both oxygen and glucose they are unable to induce VEGF, presumably because they are metabolically unable to mount a reaction requiring protein synthesis [as is hypoxia-induced VEGF expression (2)]. Since sustained nonperfusion will eventually lead to deprivation of both oxygen and glucose, the finding suggests that compensatory neovascularization may take place as long as the tissue is not overstressed. Interestingly, in contrast with the effect of hyperoxygenation, exposing spheroids to elevated glucose concentration (up to 10 g/liter) did not abolish the expression of VEGF in the inner cell layers (data not shown).

A dramatic drop in levels of VEGF mRNA was observed during *in vivo* neovascularization of implanted spheroids, specifically in regions already invaded by blood vessels (Fig. 4). These findings illustrate that the overall angiogenic output of tumor cells is adjustable and continually modulated according to the degree of tissue stress. Only a fraction of the angiogenic output is determined by the genetic program of the tumor cells.

Finally, a major research objective in the angiogenesis field is to target tumor angiogenesis. To this end, it is essential to identify the angiogenic factor(s) produced by each particular tumor. We argue that, irrespective of the identity of the angiogenic factors that may have been activated during tumor progression, any attempt to inhibit tumor neovascularization needs to consider stress-induced VEGF activity. In fact, we find that the level of VEGF mRNA induced by stress is exceedingly higher than the level dictated by the genetic program of the highly angiogenic glioma cells (Fig. 4). These observations are not a peculiarity of gliomas, as similar results were also obtained with multicell spheroids composed of lung carcinoma cells (data not shown).

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