Along with the invariant residue 25N, these amino- and carboxy-terminal regions form an important part of the TCR recognition site of the toxin molecule. □

Received 20 July; accepted 13 September 1992.

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ACKNOWLEDGEMENTS. We thank S. Swaminathan for communication of results before publication. A. Townsend for the F5 β-chain clone, L. Teyton for assistance with baculovirus and K. Ames for technical support. This work was supported by grants from the NIH, Concern Foundation for Cancer Research, Health Research Council of N.Z., N.Z. Lotteries Board and Auckland Medical Research Foundation. N.R.J.G. Is a Scholar of the Leukemia Society of America.

Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis

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INEFFICIENT vascular supply and the resultant reduction in tissue oxygen tension often lead to neovascularization in order to satisfy the needs of the tissue[1]. Examples include the compensatory development of collateral blood vessels in ischaemic tissues that are otherwise quiescent for angiogenesis and angiogenesis associated with the healing of hypoxic wounds[2]. But the presumptive hypoxia-induced angiogenic factors that mediate this feedback response have not been identified. Here we show that vascular endothelial growth factor (VEGF; also known as vascular permeability factor) probably functions as a hypoxia-inducible angiogenic factor. VEGF messenger RNA levels are dramatically increased within a few hours of exposing different cell cultures to hypoxia and return to background when normal oxygen supply is resumed. *In situ* analysis of tumour specimens undergoing neovascularization show that the production of VEGF is specifically induced in a subset of glioblastoma cells distinguished by their immediate proximity to necrotic foci (presumably hypoxic regions) and the clustering of capillaries alongside VEGF-producing cells.

To investigate a natural situation of imbalanced vascularity, we analysed the angiogenesis-dependent growth of glioblastoma tumours[3]. Glioblastoma multiforme (gm), a malignant human primary intracranial brain neoplasm, seemed most suitable for this type of analysis because in these rapidly growing tumours the development of the tumour vasculature is often unable to meet the perfusion demands imposed by the rapid expansion of the tumour. The resultant oxygen and nutritional deprivation induces extensive necrosis. These changes are accompanied by marked, almost pathognomonic proliferation of endothelial cells, with the formation of vascular glomeruli[4,5]. We reasoned that a hypoxia-inducible angiogenic factor will be predominantly expressed in a restricted subpopulation of tumour

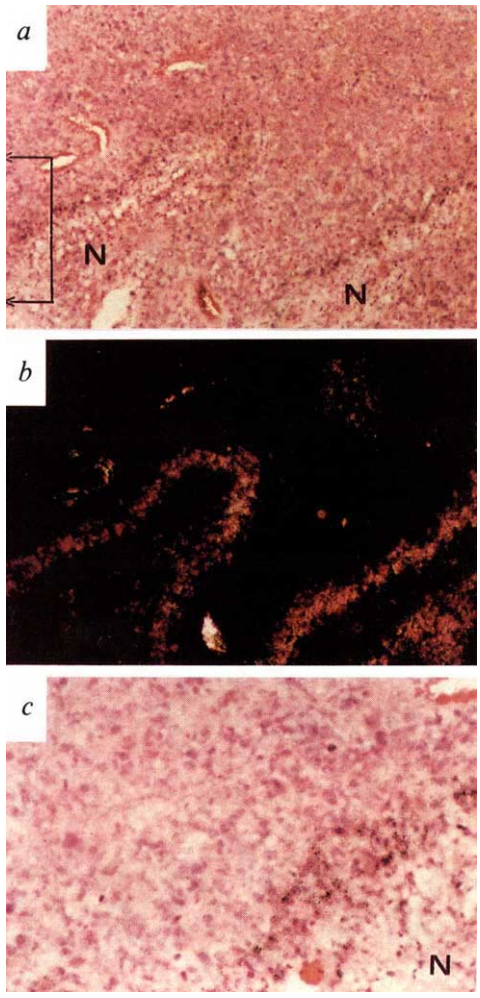

FIG. 1 Specific expression of VEGF mRNA in the periphery of necrotic areas of a glioblastoma tumour. A thin section of gm biopsy hybridized *in situ* with a VEGF-specific probe. *a* and *b*, Bright- and dark-field images, respectively; *c*, enlargement of the area boxed in *a*. Sections were counterstained with haematoxylin and eosin. Note that VEGF-expressing cells are localized alongside the edges of necrotic regions (N).
METHODS. *Tumour specimens*: Specimens of gm were obtained immediately after surgical removal for histopathological diagnosis. The remainder of the tissue was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline. Fixed specimens were incubated in 0.5 M sucrose in phosphate-buffered saline before embedding in Tissue-Tek OCT embedding medium (Miles Scientific) and *in situ* hybridization. Routinely processed formalin-fixed, paraffin-embedded specimens were also taken from the Department of Pathology, Hadassah Hospital, and processed for *in situ* hybridization, which has been described[25]. Autoradiographic exposure was for 5-9 days. Control hybridizations with a riboprobe in the 'sense' orientation were done for each probe. Hybridization probes were DNA fragments cloned into the polylinker of a PBS vector (Stratagene). These were a 590-bp-long cDNA fragment that included most of the coding region of human $VEGE_{165}$; a 1.8-kb cDNA fragment containing roughly the 3′ two-thirds of the coding region as well as the entire 3′untranslated region of mouse VEGF. (The human and mouse VEGF probes detected only the VEGF bands expected in preliminary genomic DNA blotting experiments and gave identical hybridization signals in the *in situ* hybridizations.) Constructs in the PBS vector were linearized by digestion with the appropriate restriction endonuclease to allow synthesis of a ^{35}S-labelled complementary RNA in either the antisense or sense orientation (using T3 or T7 RNA polymerase, respectively). RNA was fragmented by mild alkaline treatment before use as probes for *in situ* hybridization.

cells, reflecting the localization of hypoxic microregions. We therefore anticipated that by analysing these tumours *in situ* with respect to the spatial pattern of expression of candidate genes, hypoxia-inducible angiogenic factor might be revealed. VEGF was a particularly attractive candidate as this heparin-binding protein has angiogenic activity in the cornea and in the chorioallantoic membrane[6,7]; also, the secreted protein is a mitogen with a target-cell specificity restricted to vascular endothelial cells[8-10].

Biopsy specimens, confirmed as gm on the basis of the usual diagnostic criteria[11], were obtained shortly after surgical removal, immediately withdrawn into a fixative, and processed for *in situ* hybridization analysis using VEGF-specific, ^{35}S-labelled antisense ribopobes. *In situ* analysis of mRNA was preferred over *in situ* immunodetection of the encoded protein because the localization of the mRNA unequivocally identifies the producer cells, whereas VEGF might also be secreted and sequestered at some distance from these cells[8-10,12]. In Figs 1 and 2, mRNA is seen to be mainly produced in a small fraction of tumour cells, arranged in a stripe-like pattern alongside the periphery of necrotic regions. The highest mRNA levels were detected in tumour cells juxtaposed to cells undergoing necrosis, that is, in cells that presumably experience the most severe hypoxia.

To identify the molecular species of VEGF produced in the tumour, we extracted RNA from gm biopsies from three patients, reverse-transcribed the RNA and amplified the complementary DNA by polymerase chain reaction, using oligonucleotides derived from external exons shared by all differentially spliced VEGF mRNA species (oligonucleotides corresponding to amino acids 92-98 and to the six carboxy-terminal amino acids, respectively)[13]. The principal amplified species detected was a 225-

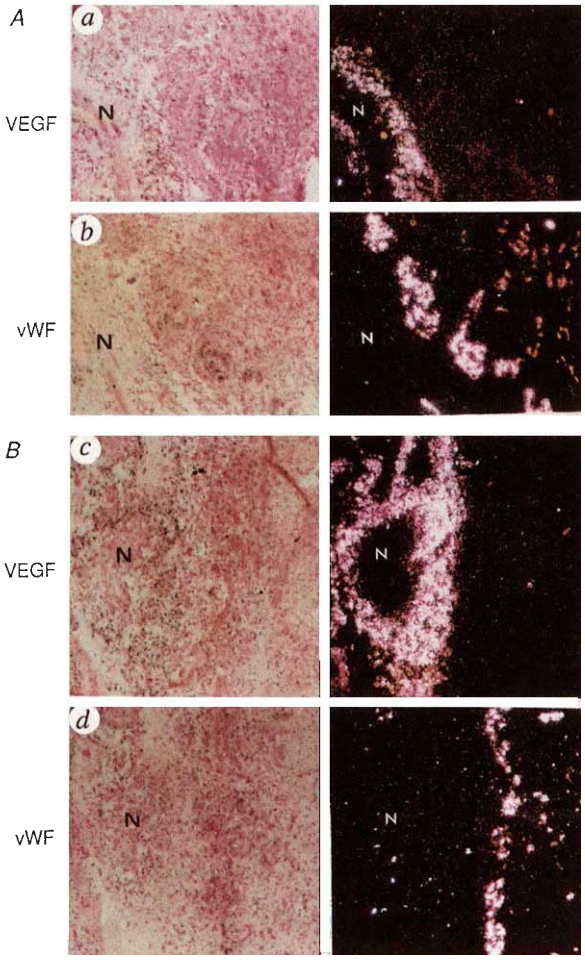

FIG. 2 Clustering of endothelial cells alongside VEGF-producing cells. 10 μm-thick sections of a gm tumour were hybridized *in situ* with VEGF-specific probe (*a*, *c*); adjacent serial sections were hybridized with a vWF-specific probe (*b*, *d*; an *ECo*RI–*Sal*I fragment derived from a human von Willebrand factor cDNA clone[26]). *A* and *B* are representative examples of necrotic islets from different regions of the same tumour. The symbol *N*, depicting the necrotic centre, also serves as a reference point to orient bright- and dark-field images (left ad right columns, respectively) and the two adjacent sections. Note that capillary bundles (vWF positive cells) are localized alongside VEGF-producing cells.

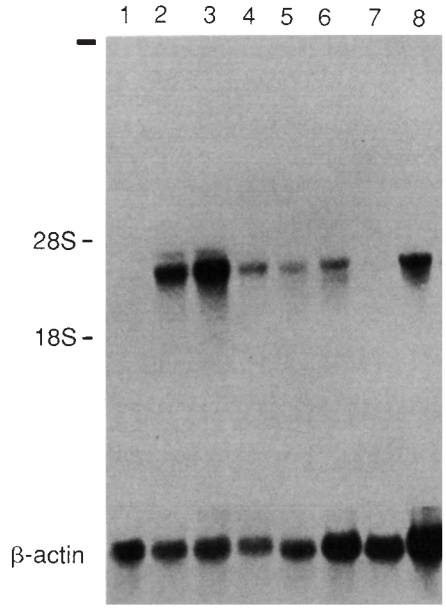

FIG. 3 VEGF expression in cell cultures is hypoxia-inducible. Cultured cells were exposed to different oxygen tensions for 18 h. The cells were collected and their RNA extracted, electrophoresed and blot-hybridized with a VEGF-specific probe. Lanes: 1, C6 cells, normal oxygen; 2, C6 cells, 95% nitrogen; 3, C6 cells, catalyst-induced anoxia; 4, same as in lane 3, followed by 8 h incubation under normal oxygen; 5, C6 cells, 20 h growth under normal oxygen in the presence of 10 μg ml^{-1} cycloheximide; 6, C6 cells, grown in the presence of 10 μg ml^{-1} cycloheximide, 2 h in normal oxygen and 18 h in catalyst-induced anoxia; 7, L8 cells, normal oxygen; L8 cells, catalyst-induced anoxia. Migration of ribosomal RNA markers (28S and 18S) is indicated.

METHODS. Cell growth under hypoxic conditions: Cell lines used were: C6, a clonal glial cell line derived from a rat glial tumour[27] and L8, a myogenic cell line isolated from primary rat skeletal muscle[28]. C6 cells were grown in Dulbeco-modified Eagle's medium supplemented with 5% FCS. L8 cells were grown in Waymouth medium supplemented with 10% FCS. Two sets of conditions were used to produce an anaerobic atmosphere: (1) cultures of nearly confluent cells were exposed to an atmosphere of 95% nitrogen and 5% CO_2; (2) cultures were incubated in GasPak Plus anaerobic culture chamber (BBL Microbiology Systems) using hydrogen and a palladium catalyst to remove all traces of oxygen. Exposure to hypoxic conditions lasted 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[29]. RNA was denatured in glyoxal and electrophoresed through a 1.0% agarose gel, then transferred onto a nylon-based membrane (GeneScreen Plus, NEN) by capillary blotting and hybridized with the indicated probes. cDNA fragments were labelled with ^{32}P by randomly primed DNA synthesis. For standardization, filters were rehybridized with a β-actin-specific probe[30]; the induction index was calculated from densitometric tracings of the autoradiogram, corrected for loading differences as determined by rehybridization with a β-actin-specific probe.

base-pair fragment corresponding to the mRNA encoding $VEGF_{165}$ (data not shown): $VEGF_{165}$ is a secreted form of the growth factor and stimulates proliferation of endothelial cells[14]. The potential targets of VEGF is the population of nearby cells expressing VEGF receptors. VEGF receptors are widely distributed on all vascular endothelial cells of adult rat tissues, including quiescent endothelium, but are not found on non-endothelial cells[14,15]. To visualize blood capillaries better we hybridized adjacent serial sections with a von Willebrand factor (VWF)-specific probe which exclusively identifies endothelial cells irrespective of their proliferation status. In Fig. 2, capillary bundles are seen to be preferentially clustered alongside the stripes of VEGF-expressing cells. Immunohistochemical labelling has shown that VEGF, which is produced elsewhere in the tumour, is concentrated in tumour blood vessels[12]. It is therefore tempting to speculate that the proximity of a high concentration of capillaries to sites of VEGF production is the result of a local angiogenic response elicited by the angiogenic factor.

The marked differences in steady-state levels of VEGF mRNA among otherwise indistinguishable tumour cells (Figs 1, 2) can be correlated with their proximity to necrotic centres, where oxygen supply is minimal. We have interpreted this observation to mean that VEGF could be specifically induced in response to hypoxia. It is equally possible, however, that VEGF is induced by factor(s) released from necrotic cells. To distinguish between these two possibilities, we determined whether VEGF expression is regulated by oxygen. Glioma cells were grown under normoxic and hypoxic conditions, and levels of VEGF mRNA were subsequently measured by RNA-blot analysis. Steady-state levels of VEGF mRNA were significantly increased within 18 h of growth under low oxygen tensions (Fig. 3). A 13-fold induction was measured under complete anoxia (as achieved by oxidation with a palladium catalyst). The increase in VEGF mRNA levels was reversible. Upon re-exposure of cells to normal oxygen tension, VEGF expression resumed its low constitutive level (Fig. 3). No evidence for cell death could be detected in cultures exposed to anoxia, and cells continued to proliferate at a normal rate following re-exposure to normal oxygen tension.

These findings support the thesis that the induction of VEGF in gm tumours occurs in response to hypoxia. They also indicate that the rate of release of angiogenic factors by tumour cells might in general be variable, being constantly adjusted according to the changes in the cell microenvironment.

To determine whether VEGF is hypoxia-inducible in other cell types, we did a similar experiment with a cell line of skeletal muscle myoblasts (L8). As shown in Fig. 3, VEGF was also hypoxia-inducible in these cells (5-fold induction after standardization for RNA loads). Similar levels of induction were also observed in a fibroblast (mouse L-cells) line and in primary cultures of cells derived from rat heart muscle (data not shown). We conclude that VEGF is hypoxia-inducible in a variety of cell types. These results suggest that, in principle, VEGF might play a part in ischaemia-induced collateralization responses taking place in normal muscle and connective tissues.

It has been proposed that basic fibroblast growth factor (bFGF) is released by tumour cells, ischaemic myocardium and other tissues in which ischaemia has caused extensive cell damage[16]. We could not detect any significant increase in steady-state levels of bFGF mRNA at sites where expression of VEGF was upregulated (data not shown). This finding does not exclude, however, the possibility that cell damage causes the release of pre-made bFGF from its stores[17].

The mechanism(s) that control responsiveness of VEGF to hypoxia are unknown. Precedents for other hypoxia-inducible genes include examples of both transcriptional activation (such as platelet-derived growth factor B-chain gene[18]) and post-transcriptional regulation by a *trans*-acting protein that physically interacts with the gene (erythropoietin for example[19]). Preliminary results with a general inhibitor of protein synthesis have indicated that VEGF induction depends on prior protein synthesis (Fig. 3). It also remains to be determined whether increased mRNA production is due to low oxygen, or is secondary to increased lactate in the environment (as in the case of angiogenesis mediated by macrophages recruited to hypoxic wounds[20]) or to accumulation of other metabolites.

Finally, VEGF has potent vascular pemeabilization activity[21,22], and hypoxaemia is associated with increased vascular permeability[23,24]. It is possible that VEGF functions as the link connecting hypoxia and increased vascular permeability. The apparent leakiness of blood vessels in gm tumours[4] could also be accounted for by the large amounts of VEGF secreted by the tumour cells. □

Received 6 May; accepted 9 September 1992.

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ACKNOWLEDGEMENTS. This work was supported by a grant from the Israeli Academy of Sciences.

Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*

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CLINICAL and experimental studies suggest that angiogenesis is a prerequisite for solid tumour growth[1,2]. Several growth factors with mitogenic or chemotactic activity for endothelial cells *in vitro* have been described, but it is not known whether these mediate tumour vascularization *in vivo*[3,4]. Glioblastoma, the most common and most malignant brain tumour in humans, is distinguished from astrocytoma by the presence of necroses and vascular proliferations[5,6]. Here we show that expression of an endothelial cell-specific mitogen, vascular endothelial growth factor (VEGF), is

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