RELATIONSHIP BETWEEN VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION AND ANGIOGENESIS AND CELL PROLIFERATION OF MALIGNANT GLIOMAS IN CHILDREN

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ABSTRACT

Objective: To investigate the relation of vascular endothelial growth factor (VEGF) expression with angiogenesis and cell proliferation in malignant glioma in children. Methods: Immunohistochemical technique was used to detect the expression of VEGF, microvessel quantity (MVQ) and PCNA Labeling Index (PCNA LI) in 33 malignant gliomas in children Results: Positive staining for VEGF was obtained in 23 out of the 33 cases (69.7%). The MVQ and PCNA LI in VEFG-positive tumors were significantly higher than those in VEGF-negative tumors ($P<0.005$). The expression of VEGF in tumor tissues was significantly correlative with MVQ and PCNA LI (r=0.52 and 0.37, respectively, $P<0.001$). Conclusion: VEGF can be synthesized in tumor cells of malignant glioma in children which might play a significant role in angiogenesis and cell proliferation in the tumor.

Key words: Glioma; Vascular endothelial growth factor, Angiogenesis, Proliferation, Children

There is mounting evidence that tumor angiogenesis is intimately related to tumor growth, invasion, metastasis and prognosis. Tumor angiogenesis is regulated by some kinds of cytokines in tissues, such as fibroblast growth factor (FGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), among which, VEGF is known to be particularly responsible for the process of brain tumor angiogenesis VEGF influence on biological properties of adult brain tumor have been reported in other articles.[1] In this study, we report on the VEGF expression and its relationship to the frequency of tumor cell proliferation and tumor vascularity in 33 childhood malignant gliomas using immunohistochemistry and antibodies to VEGF, proliferating cell nuclear antigen (PCNA) and endothelium (factor VIII).

MATERIALS AND METHODS

Tumor Tissue

Tumor specimens were obtained from a total of 33 patients with glioma who underwent surgical resection at Xijing Hospital. Histologically, the tumors consisted of 12 anaplastic astrocytomas, 16 glioblastomas, and 5 medulloblastomas. The histological diagnosis was confirmed by a neuropathologist using a portion of the original tumor tissue. Of the patients, 17 were male and 16 female with an age range from 1.5 to 15 years(mean=9.2).

Immunohistochemical Staining

All specimens were fixed with 10% formalin, embedded in paraffin and then sectioned. Immunohistochemical staining was performed on 5 μm sections using a standard avidin-biotin complex (ABC) peroxidase technique. The sections were incubated for 1 hour at 55-60°C and deparaffinnized and cleared with xylenes, through a series of graded alcohols and hydrated with deionized water. Endogenous peroxidase activity was quenched with 2% hydrogen peroxide in phosphate-buffered saline (pH 7.4) for 7 minutes. A primary antibody to VEGF (a polyclonal antiserum, from Santa Cruz Biotechnology Co.) or PCNA (a monoclonal antibody, from Dako Co.) was applied to the slides in a 1: 20 dilution for 24 hours at 4°C. The biotinylated secondary antibody and ABC complex were applied to the slides in sequence for 30 minutes at 37°C. The

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peroxidase of activity was made visual with chromogenic precipitate diamino-benzidine. Counter staining was performed with hematoxylin. Negative controls were carried out by substituting the primary antibody with PBS. For evaluation of VEGF expression a scoring system corresponding to the percentage of positive cells. 0=0% positive cells; 1=<25% positive cells; 2=26–50% positive cells; 3=51—75% positive cells; 4=>75% positive cells). A score no less than 2 was regarded as the value of a positive immunohistochemical assay. Determination of tumor cell PCNA LI was scored by selecting the maximally immunostained areas and counting PCNA-positive and –negative tumor cells at × 400 magnification and with an eyepiece grid. All reactive cells were counted as positive regardless of the intensity of the staining. In each case, 500–1000 cells were counted and the fraction of positive cells ratio was determined.

Microvessel Quantity

Intratumoral MVQ was highlighted by immunostaining using antihuman factor VIII related-antigen monoclonal antibody and LSAB Kit (Dako Co.). The MVQ was determined by a method described by Maeda, et al.[2] Briefly, the tumor sections were scanned under low and intermediate powers to determine the area of most intense neovascularization (hot spots). Then MVQ of five such areas in each tumor was counted under a 200-fold magnification. The average count from the five areas was used as the final MVQ.

Statistical Analysis

Quantitative data were expressed as $\bar{\chi} \pm s$, analysis of quantitative data was performed using Student's test and linear correlation.

RESULTS

VEGF Expression and MVQ

Positive staining for VEGF was mainly identified in the cytoplasma of tumor cells. A weak positive VEGF staining was also seen on endothelial cells. Twenty-three out of the 33 cases (69.7%) of malignant glioma exhibited positive expression of VEGF. The tumors were found to be quite heterogeneous in MVQ. The areas of high vascularization occurred most frequently at the margins of the tumors. The mean MVQ for all tumors was 52.9 ± 30.5. The MVQ in VEGF-positive tumors (61.7 ± 22.9) was significantly higher than that in VEGF- negative tumors (32.6 ± 19.4)(t=3.50, P<0.001). The expression of VEGF is significantly correlated to MVQ (R=0.52, p<0.001).

VEGF Expression and Tumor Cell Proliferation

PCNA has proved to be a sensitive marker of tumor cell proliferative potential. Proliferating tumor cells were easily identified by nuclear immunostaining with the PCNA antibody. The mean PCNA LI of all tumors was 44.3% ± 35.8%. The PCNA LI in VEGF-positive tumors (52.6% ± 24.9%) was significantly higher than that in VEGF-negative tumors (25.2% ± 18.2%) (t=3.12, P<0.005). The expression of VEGF was significantly correlated to PCNA LI (r=0.37, P<0.001).

MVQ and PCNA LI

Tumor cell proliferation, as assessed by the PCNA LI in the maximally immunostained areas, was correlated with vascularity, measured in the vascular hotspots. There was no correlation between tumor cell MVQ and PCNA LI (r=0.08, P>0.05).

DISCUSSION

Bruce and Criscuolo[3] first demonstrated that glioma cells can synthesize and secret VEGF. There are two known receptors for VEGF, flt and flk, both belong to the receptor tyrosine kinase family. The mRNA encoding the flt and flk in the endothelial cells of tumors has been found and confirmed. VEGF secreted by tumor cells is a powerful endothelial cell-specific mitogen that acts on endothelial cell proliferation, induces tumor angiogenesis in a paracrine fashion.[4] In this study we have examined the relationship of VEGF expression to tumor cell proliferation and MVQ in childhood malignant gliomas. The present results indicate that the MVQ in VEGF-positive tumors is significantly higher than that in VEGF-negative tumors (P<0.005), and the expression of VEWGF is significantly correlated to MVQ (r=0.52, P<0.001). These findings are consistent with the above results. It demonstrated that childhood malignant glioma cells could produce VEGF, which might play a significant role in angiogenesis and cell proliferation.

Neovascularization is needed in the tumor for the growth of solid tumors beyond a size 2–3 mm in diameter, with a cell number magnitude of 10^7. Tumor angiogenesis supports tumor enlargement, because the growth of solid tumors needs an adequate vascular network for supply of oxygen, nutrients and removal of waste products. The transition from limited to rapid tumor growth often accompanies the transition from the prevascular to the vascular phase. It has been established that tumor cell proliferation decreases with increasing distances of them from the blood vessels. Experimental study demonstrated that angiogenesis inhibitors administration can not

suppress tumor cell growth *in vitro*, but could be *in vivo*, which indicated that inhibitors induced angiogenesis suppresses tumor growth *in vivo*.[5] These data suggested that tumor growth is dependent upon angiogenesis. We found that PCNA LI in VEGF-positive tumors is significantly correlated to PCNA LI ($r=0.37$, $P<0.001$). VEGF receptors were not detected in glioma cells by Weindel and collaborators.[6] From these results, we conclude that VEGF may play a promoting role in cells proliferation of childhood malignant glioma.

Although the MVQ of tumors is closely related to tumor cells proliferation, in this study it seems that there is no correlation between MVQ and PCNA LI ($r=0.08$, $P>0.05$) in childhood malignant glioma, which is consistant with the results in human breast cancer published from Fox, et al.[7] and contrary to the results from Vermeulen, et al. in human colorectal adenocarcinomas.[8] Taken together, the data suggested that tumor angiogenesis and tumor cell proliferation may be regulated by different mechanisms, which remains to be clarified.

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