Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 7748–7752, October 1988
Medical Sciences

Endothelial cell hyperplasia in human glioblastoma: Coexpression of mRNA for platelet-derived growth factor (PDGF) B chain and PDGF receptor suggests autocrine growth stimulation

(glioblastoma multiforme/*in situ* hybridization/endothelial cells)

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Communicated by Max D. Cooper, June 3, 1988

ABSTRACT **The genes for platelet-derived growth factor (PDGF) A chain, B chain/c-*sis*, and the PDGF receptor are expressed in human malignant glioma cell lines. In the present investigation we have studied the expression of these genes in biopsy specimens from human glioblastomas. Hyperplasia of the vascular endothelium is a prominent characteristic of human glioblastoma multiforme and simian sarcoma virus-induced gliomas in primates. RNA transfer blot analysis of biopsies from glioblastoma multiforme showed transcripts for PDGF A and B chains and the PDGF receptor. Tissue sections from this tumor examined by *in situ* hybridization techniques revealed that the proliferating vascular endothelial cells contained large quantities of mRNA for PDGF B chain/c-*sis* and its receptor and, to a lesser extent, for PDGF A chain. In contrast, the tumor cells expressed more mRNA for PDGF A chain than for PDGF B chain and PDGF receptor. The latter two were also expressed at higher levels in glioma cells than in glial cells of nontumorous human brain tissue. Thus, an autocrine stimulation by the PDGF B chain/c-*sis* product via its receptor, evoked by interaction with surrounding glioma cells, could be the mechanism behind the pathological proliferation of endothelial cells characteristically found in this type of malignancy.**

There is a growing body of evidence that an autocrine production of growth factors may operate in neoplastic transformation and tumor development (1). One of the best-studied examples of an autocrine mitogen is platelet-derived growth factor (PDGF), which is the major growth factor in serum for connective tissue- and glia-derived cells in culture (2). The molecule is made up as a dimer of A and B chains (3, 38). The B chain gene is the normal cellular homologue to the oncogene v-*sis* of simian sarcoma virus (SSV) (4, 5). SSV transformation of cultured cells is executed by a PDGF-like growth factor, which is homologous to a homodimer of B chains (6) and which exerts its function by means of binding to the PDGF receptor of the producing cell (7, 8). Circumstantial evidence that a similar mechanism may be involved in the early stages of the development of malignant tumors is provided by the finding that SSV causes fibrosarcomas and malignant gliomas when experimentally administered in newborn marmosets (9). Gliomas induced in this way show proliferation of endothelial cells, resembling what is found in spontaneous human malignant gliomas (10).

In view of the finding that SSV causes malignant brain tumors, it should be of interest to know whether an autocrine growth stimulation, involving PDGF-like growth factors, operates in the pathogenesis of human glioma. Previous studies have shown that many human malignant glioma cell lines express the PDGF genes (11) and produce PDGF-like growth factors (12); some of the cell lines express PDGF receptors as well (11). However, permanent cell lines are selected for long-term growth in culture and cannot be considered true representatives of the tumor cell population *in vivo*. In the present study we have used nucleic acid hybridization techniques to analyze the expression of PDGF A chain, PDGF B chain/c-*sis*, and PDGF receptor mRNA in biopsies of human glioblastoma multiforme. By using *in situ* analysis we were able to demonstrate an expression of all three genes in glioma cells, A chain mRNA being predominantly expressed in areas of tightly packed anaplastic cells. The most conspicuous feature, however, was a strong coexpression of PDGF B chain/c-*sis* and PDGF receptor mRNA in hyperplastic endothelium. This finding suggests a mechanism involving an autocrine activation of the PDGF receptor in endothelial cell proliferation and possibly tumor neovascularization.

MATERIALS AND METHODS

Biopsy Material. Fresh human material was obtained from three cases of glioblastoma multiforme. Parts of the fresh material were immediately selected for mRNA preparation and RNA transfer blot analysis. Normal rat and pig brain tissues were used as controls in this analysis. For *in situ* hybridization, the rest of the human material was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Rat and pig tissues were frozen and cryostat sections were fixed in 4% paraformaldehyde. All sections were stained with hematoxylin/eosin and/or Weigert–van Gieson stain for light microscopic evaluation. Serial sections were used for *in situ* hybridization and immunohistochemical staining. Formalin-fixed human cerebral tissue from a case of arteriovenous malformation was used as additional control tissue for the *in situ* hybridization.

RNA Transfer Blot Analysis. Total cellular RNAs from fresh tumor biopsies as well as from rat and pig brain tissues were isolated by using the LiCl/urea method (13). Poly(A)$^+$ RNA was selected by one cycle on oligo(dT)-cellulose (type 7, Pharmacia) and precipitated with sodium acetate, pH 5.2/ethanol. Aliquots of RNA were denatured for 5 min at 65°C in 50% formamide/2.2 M formaldehyde/20 mM 3-(*N*-morpholine)propanesulfonic acid, pH 7.0/5 mM sodium acetate/1 mM EDTA/10% glycerol. Size fractionation was made on 0.8–1% agarose/2.2 M formaldehyde slab gels. RNA was then transferred to nitrocellulose filters (BA 85, Schleicher & Schüll), which were baked at 80°C under

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Abbreviations: PDGF, platelet-derived growth factor; SSV, simian sarcoma virus; UEAI, *Ulex europaeus* agglutinin I lectin; GFAP, glial fibrillary acidic protein; TGF, transforming growth factor.

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vacuum for 2 hr. The filters were hybridized to ^{32}P-labeled 1.3-kilobase (kb) PDGF A chain cDNA (14) fragment, 2.7-kb PDGF B chain/c-*sis* cDNA (ref. 15; kindly provided by F. Wong-Staal), and 1-kb PDGF receptor cDNA (39). The hybridization was performed (16) in 50% formamide/750 mM NaCl/75 mM sodium citrate/5 × Denhardt solution (1× Denhardt solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.1% $NaDodSO_4$/0.2 mg of salmon sperm DNA per ml for 24–48 hr at 42°C, washed in 300 mM NaCl/30 mM sodium citrate/0.5% $NaDodSO_4$ for 10 min, and followed by washing in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ for 1–2 hr at 56°C. The filters were then exposed on Kodak XAR-5 film or Amersham Hyperfilm-MP with intensifying screens for 5–7 days at −70°C.

***In Situ* Hybridization.** Formalin-fixed paraffin blocks of biopsied tissues were sectioned, deparaffinized, and digested with 0.1 mg of proteinase K per ml (Sigma) prior to hybridization. *In situ* hybridization was performed as described elsewhere (17). ^{35}S-labeled RNA probes were transcribed *in vitro* from plasmids containing the following DNAs: 1.3-kb *Eco*RI fragment of cDNA for A chain (14), 2-kb *Bam*HI fragment of a human B chain cDNA (C.B., A. Johnsson, T. Knott, and J. Scott, unpublished data), and 1-kb *Pst* I fragment of a cDNA corresponding to the external domain of the human PDGF receptor (39), all subcloned in pGEM vector (Promega Biotec, Madison, WI). The 1.9-kb *Bam*HI fragment of the cDNA for human β-actin (18) was also subcloned in SP65 vector (Promega Biotec). *In vitro* transcription was done as described (17). Labeled probe (1×10^6 cpm per section) was mixed with hybridization buffer and added to the tissue sections on slides. Hybridized preparations were autoradiographed with NTB 2 nuclear track emulsion (Kodak) diluted 1:1 with distilled water. After exposure for 5 days at 4°C, slides were developed in Dektol (Kodak) developer at 15°C for 4 min, rinsed in distilled water, fixed for 5 min, soaked in distilled water for 5 min, and air-dried. Slides were counterstained with Mayer hematoxylin/eosin. Control slides include the following: (*i*) positive and negative cultured control cells for each probe were used to check the specificity [Chinese hamster ovary (CHO) cells transfected with A chain or B chain DNA (A. Östman, unpublished data) and human foreskin fibroblasts, line AG1523 (Coriell Institute for Medical Research, Camden, NJ), known to express the PDGF receptor, as positive cells for the respective probes, and nontransfected CHO cells as negative controls]; (*ii*) to exclude cross-hybridization between A and B chain probes, the A chain probe was used on B chain transfected CHO cells and vice versa; (*iii*) on each tissue slide, pretreatment with RNase prior to hybridization was performed to check that the probe was hybridized with cellular RNA; (*iv*) an RNA probe transcribed in opposite direction from the plasmid with the B chain insert was used to estimate the background; (*v*) all tissues were checked for the presence of cellular mRNA by hybridizing with the RNA probe for human β-actin.

Immunohistochemistry. Avidin-biotin-coupled immunoperoxidase staining was performed on formalin-fixed, paraffin-embedded material by using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the vendor's description. For staining of endothelial cells, *Ulex europaeus* agglutinin I lectin (UEAI) and biotinylated rabbit anti-*Ulex* antibody obtained from Vector Laboratories were used at 1:800 and 1:100 dilution, respectively. For visualization of the glial fibrillary acidic protein (GFAP), a rabbit anti-GFAP antibody (Dakopatts, Hägersten, Sweden) was used at 1:800 dilution. Lymphoid cells were identified by staining with anti-leukocyte common antigen (Dakopatts) at 1:5 dilution, and muscle cells were identified by a monoclonal anti-desmin antibody (Monosan, Uden, The Netherlands) at 1:10 dilution. In each assay the following control steps were included: normal rabbit serum was substituted for the primary antibody (negative control) and previously known positive tissues were included.

RESULTS

RNA Transfer Blot Analysis. Poly(A)$^+$ RNAs from three cases of glioblastoma multiforme were hybridized with the PDGF A chain, B chain/c-*sis*, and PDGF receptor cDNAs. All were found to give strong positive signals (Fig. 1). Poly(A)$^+$ RNAs from cerebral tissues of normal animals showed significantly lower signals. Thus, pig but not rat brain mRNA gave a very weak PDGF A chain signal and both of them showed weak bands for PDGF B chain/c-*sis*. No PDGF receptor transcript was demonstrated. Since we could show DNA bands comparable to humans in Southern blot analysis using these cDNA probes (result not shown), we assumed that their transcripts would be detected under the stringency employed. Case 3 in Fig. 1 was selected for *in situ* hybridization in order to assign the gene expressions to different cell types present in glioma tissue. This glioblastoma multiforme contained areas of extensive vascular endothelial proliferation with glomeruloid structures.

***In Situ* Hybridization.** ^{35}S-labeled PDGF A and B chain and receptor RNA probe were used for *in situ* hybridization. The localization of grains to different cell types was aided by comparison with immunohistochemical stainings of serial sections (Fig. 2). The mRNA levels were estimated by counting the number of grains per nucleus of glioma or glial cells and endothelial cells, respectively. Table 1 shows the

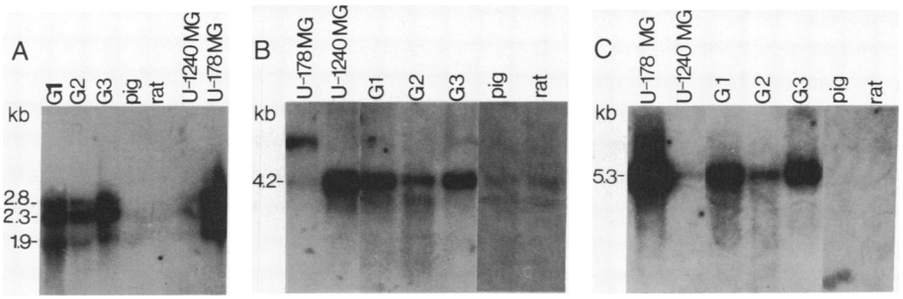

FIG. 1. Transfer blot analysis of poly(A)$^+$ RNA (15 μg per lane) prepared from glioblastoma multiforme (G1–G3) as well as from normal rat and pig brain tissues. Filters were hybridized to ^{32}P-labeled human PDGF A chain (*A*), PDGF B chain (*B*), and PDGF receptor (*C*) cDNA probes. The A chain probe hybridized to three transcripts (2.8, 2.3, and 1.9 kb), the B chain probe hybridized to one 4.2-kb and a smaller 3.7-kb transcript, and the PDGF receptor hybridized to a single 5.3-kb transcript. For comparison, poly(A)$^+$ RNA from glioma cell lines (10 μg per lane) was used. U-178 MG expresses a high amount of PDGF A chain and receptor, and U-1240 MG expresses a high amount of PDGF B chain mRNA (11). The 5.3-kb transcript on lane U-178 MG, when hybridized with the B chain probe, is a signal left from the prior hybridization with the PDGF receptor probe.

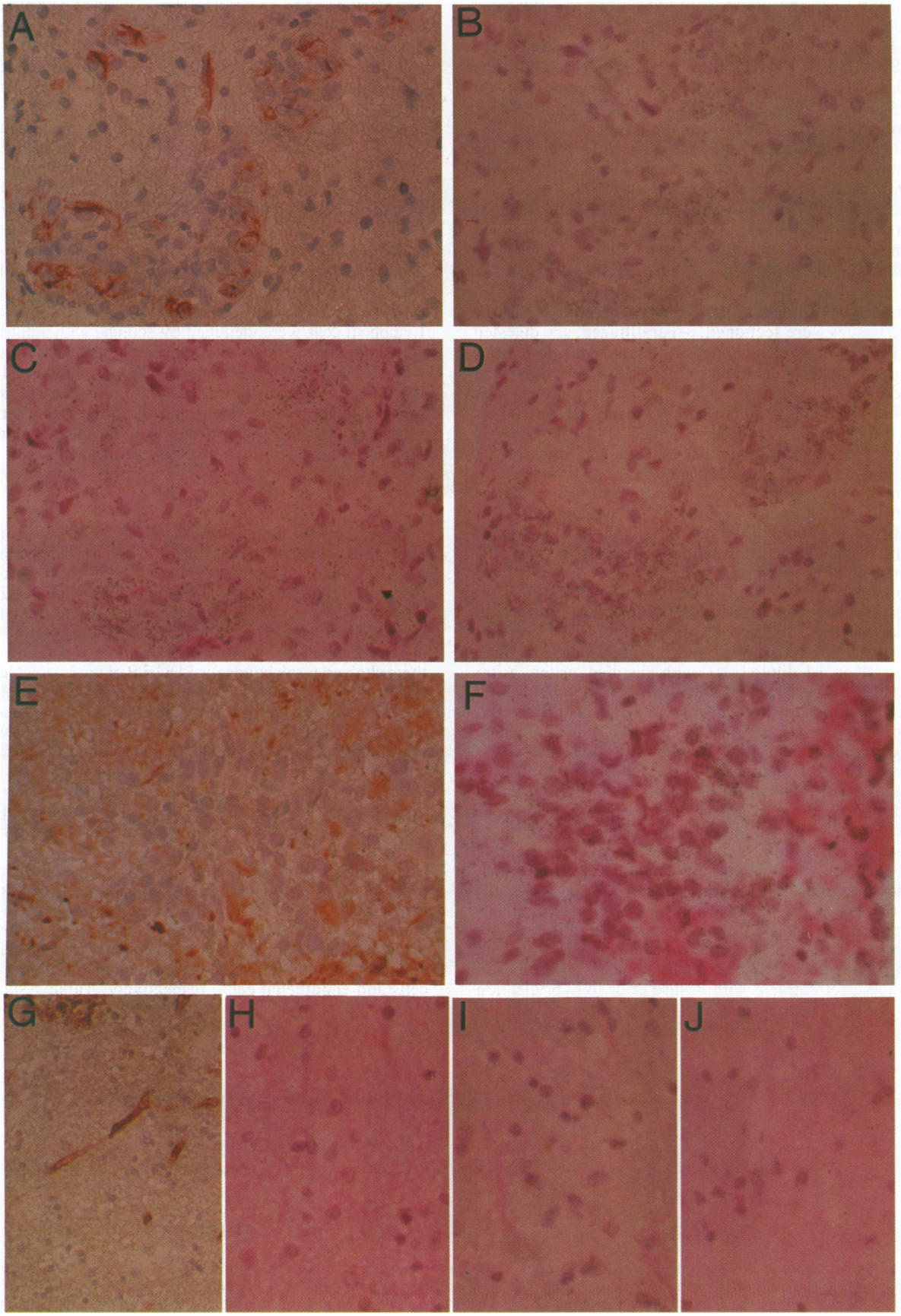

FIG. 2. (*Legend appears at the bottom of the opposite page.*)

result of hybridization with the three probes applied to sequential sections of one glioblastoma multiforme that was extremely rich in endothelial proliferations. Nontumorous human cerebral tissue adjacent to the arteriovenous malformation was hybridized simultaneously. The same distribution of grains between endothelium and glial or glioma cells was seen with repeated hybridizations to different sections from the same biopsy specimen and with hybridization to another case of glioblastoma multiforme with extensive endothelial proliferation (result not shown). PDGF B chain (Fig. 2*C*) and PDGF receptor mRNA (Fig. 2*D*) were preferentially localized to the proliferating endothelial cells in small capillaries and glomeruloid structures. Endothelial cells in nontumorous human brain tissue (Fig. 2 *H–J*) as well as in normal pig cerebrum (result not shown) gave only background levels of the same RNA species. A few endothelial cells in nontumorous human control tissue showed a slightly higher than background level of the signal for PDGF receptor mRNA (Fig. 2*J*), which was, however, not statistically significant. In the glioblastoma tissue, PDGF B chain and PDGF receptor mRNA were not restricted to endothelial cells but were also localized to malignant glioma cells since the number of grains per nucleus for both mRNA types was two to three times higher in glioma cells than in normal glial cells of the human tissue. PDGF A chain mRNA was found in proliferating endothelial cells (Fig. 2*B*) and in glioma cells at a higher level than in corresponding normal cells. The expression of the A chain gene seemed especially high in areas of tightly packed anaplastic glioma cells (Fig. 2*F*), some of which were negative for GFAP (Fig. 2*E*).

Immunohistochemical Staining. To clearly distinguish between the proliferating endothelial cells and glioma cells, we have performed immunohistochemical staining using UEAI, which binds to blood group H antigen on the surface of endothelial cells (19–21). Bound lectin was visualized by biotinylated rabbit anti-lectin antibody sequentially coupled with avidin·biotin·peroxidase complex. The lectin distinctly bound to normal endothelial cells in the control tissues from pig and rat cerebrum as well as to the endothelium in human cerebral tissue adjacent to arteriovenous malformations. In the glomeruloid structures in glioblastoma multiforme, the inner layer of endothelial cells, which faces the small capillary lumens, showed more intense and distinct staining than did the outer layers, facing adventitia and glioma cells, as has been reported by others (20, 22). However, it is likely that the proliferating cells inside the glomeruloid tufts all represent endothelial cells since they were distinctly surrounded by collagen lamina visualized by Weigert–van Gieson staining (result not shown). No cells around the glomeruloid structure in glioma tissue were stained by anti-desmin antibodies (result not shown). This contrasted with the vascularization in the malformed brain used as a nontumorous control, which showed a considerable amount of positively stained smooth muscle cells. Anti-GFAP antibodies were used to visualize glioma cells as well as glial cells in normal brain tissue. Some areas with tightly placed anaplastic glioma cells, expressing the PDGF A chain gene (Fig. 2*F*), did not contain detectable GFAP (Fig. 2*E*; ref. 23). Control staining of the same area with UEAI was negative (result not shown). Additional staining with leukocyte common antigen was used to identify lymphoid cells in the glioma specimen. However such cells were few and localized only in the periphery of some large vessels (result not shown).

Table 1. Grains per nucleus in tissues hybridized *in situ* to PDGF A chain, PDGF B chain/c-*sis*, and PDGF receptor RNA probes

		Endothelial cells		Surrounding cells (glioma or glial cells)	
Tissue	Probe	*n*	Grains, no. per cell	*n*	Grains, no. per cell
Glioma	A chain	101	4.5 ± 2.7	101	9.4 ± 4.8
Control*	A chain	53	0.8 ± 1.0	94	0.3 ± 0.8
Glioma	B chain	173	9.6 ± 7.4	111	3.1 ± 1.8
Control*	B chain	52	1.3 ± 1.4	101	1.0 ± 1.2
Glioma	Receptor	158	12.4 ± 6.1	124	2.6 ± 1.4
Control*	Receptor	84	1.1 ± 1.5	95	0.6 ± 1.0
Glioma	Control	100	0.7 ± 0.9†		
Control*	Control	100	0.8 ± 1.1†		

Values are expressed as mean ± SD.
*Formalin-fixed human cerebral tissue from a case of arteriovenous malformation was used as a control.
†Endothelial cells and surrounding cells are included since no significant difference in grain content was seen between those cells. A B chain probe transcribed in the opposite (sense) direction was used as the control probe to estimate background.

DISCUSSION

This study has presented evidence for the *in vivo* expression of the genes encoding PDGF and its receptor in human malignant glioma. Thus, transcripts of proper sizes were found on transfer blots of mRNA preparations from three tumors and at considerably higher levels than found in normal rat or pig cerebral tissues used as controls. *In situ* analysis revealed expression of the genes in glioma cells, in confirmation of our previous studies on a panel of continuous human glioma cell lines (11). Other studies on biopsy material have shown a high frequency of epidermal growth factor (EGF) receptor gene amplification and a corresponding high level of EGF receptor mRNA (24) in glioblastoma multiforme, and analysis of cell lines has revealed expression of transforming growth factor-α (TGF-α) mRNA in glioma cells (11). Together with the present results, these findings suggest that autocrine growth stimulation, involving TGF-α- and PDGF-dependent pathways, may be of importance in the pathogenesis of human glioma. Future studies will show if the autocrine synthesis of TGF-α and PDGF is a unique property of tumor cells in the adult brain or if growth factor production may also occur in normal cells—e.g., in normal regenerating glia. This is an interesting possibility since the blood–brain barrier may exclude these cells from exogenous sources of growth factors.

A conspicuous finding in the present investigation was the coexpression of PDGF B chain/c-*sis* and PDGF receptor mRNA in vascular endothelial cells. Proliferating endothelium is considered a hallmark of high-grade gliomas. Al-

FIG. 2 (on opposite page). Photomicrographs showing the proliferation of the vascular endothelium in a case of glioblastoma multiforme. (*A*) Immunohistochemical staining with UEAI and biotinylated rabbit anti-UEAI antibody showing a patchy membranous reaction in the glomeruloid structures of endothelial cells. (×270.) (*B*) Area corresponding to that in *A* showing positive silver grains when hybridized with PDGF A chain probe. (×270.) (*C* and *D*) The same area as in *A* and *B* showing strong signals confined to endothelial cells when hybridized with PDGF B chain and PDGF receptor probes. (×270.) (*E*) Photomicrographs of the same glioblastoma multiforme showing an area with tightly packed anaplastic glioma cells. Scattered cells stain positively by anti-GFAP antibodies. (×360.) (*F*) Area corresponding to that in *E* showing strong hybridization with PDGF A chain probe. (×360.) (*G*) Photomicrographs of human cerebral tissue adjacent to arteriovenous malformations stained with UEAI. There is a distinct positive staining of all vascular endothelial cells. (×169.) (*H–J*) The same area as in *G* hybridized with PDGF A chain, PDGF B chain, and receptor probes. Only grains at the background level are seen. However, with the PDGF receptor probe some vascular endothelial cells contain slightly more grains than glial cells. (×270.)

though the cells grow in solid, tumor-like formations, which is some cases may comprise a major part of the tumor mass, they are generally believed to constitute a nonneoplastic element. Thus, in contrast to glioma cells, the proliferating endothelial cells are diploid (25). The pathogenetic mechanism of endothelial hyperplasia in malignant glioma remains entirely obscure. An obvious possibility is that the endothelial cells respond to mitogens released from the glioma cells. One such mitogen is acidic fibroblast growth factor, which is produced by glioma cells in culture (26). Based on the present findings, we would like to suggest that endothelial cell proliferation in glioma may involve an autocrine mechanism. Previous studies on fibroblasts in culture have shown that the expression of the PDGF B chain/c-*sis* (or v-*sis*) genes in cells synthesizing the receptor confers an autonomous growth signal and a selective growth advantage, as revealed by focus formation (7, 8). By analogy, we consider it likely that autocrine activation of the PDGF receptor pathway in endothelial cells, evoked by interaction with the surrounding glioma cells, may generate a mitogenic signal that allows the cells to hyperproliferate and sometimes grow in pseudotumor formations. It is interesting that a similar mechanism has been proposed for the pathogenesis of the endothelial cell-derived Kaposi sarcoma, in this case involving a fibroblast growth factor-like factor (27).

The expression of mRNA for PDGF A and B chain and production of PDGF-like growth factor activity by regenerating endothelial cells have been established (28, 29). Endothelial cell-derived PDGF is thought to play a role in maintaining the integrity of the vascular wall as a paracrine growth factor for smooth muscle cells of the media (28–30). Previous studies have mainly concerned endothelial cells derived from large vessels; endothelial cells *per se* have generally been considered nonresponsive to PDGF. This view is supported by the absence of ^{125}I-labeled PDGF binding (31). At present, we do not know whether the expression of PDGF receptor mRNA in endothelial cells occurs only as an event specific for blood vessels in gliomas. However, our finding certainly warrants a reconsideration of endothelial cells, especially of capillary origin, as potential targets for PDGF-like growth factors, be it by means of autocrine or paracrine routes.

The progressive growth of a malignant tumor requires an adequate blood supply, which is furnished by newly formed vessels. It is generally assumed that pathological and normal neovascularization is provoked by specific angiogenic factors (32). Thus, a family of structurally unrelated peptides has been shown to be angiogenic: fibroblast growth factor (33), angiogenin (34), TGF-α (35), TGF-β (36), and tumor necrosis factor-α (37). It is interesting to speculate that also PDGF is a member of this family. An autocrine synthesis of PDGF in hyperplastic endothelium of malignant glioma may be a pathological manifestation of a normal mechanism of angiogenesis, in which cell proliferation dominates at the expense of cell migration and differentiation, required for the formation of functioning vessels.

Note Added in Proof: The existence of two separate PDGF receptor types has recently been described (40, 41). The receptor cDNA used in this work corresponds to the B-type receptor.

We thank Dr. Christer Busch for advice, Dr. Lena Claesson-Welsh for the PDGF receptor probe, Christina Bergqvist and Ulla Pihlgren for sectioning the tissues, and Pablo Lavignasse for immunohistochemical work. This work was partly supported by grants from the Swedish Cancer Society.

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