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Human brain glial cells synthesize thrombospondin

(platelet-derived growth factor)

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ABSTRACT **Thrombospondin, a 450-kDa multinodular glycoprotein with lectin-type activity, is found in human platelets, endothelial cells, fibroblasts, smooth muscle cells, monocytes, and granular pneumocytes. Thrombospondin interacts with heparin, fibrinogen, fibronectin, collagen, histidine-rich glycoprotein, and plasminogen. Recently, thrombospondin synthesis by smooth muscle cells has been reported to be augmented by platelet-derived growth factor. We present evidence that thrombospondin is present within and synthesized by astrocytic neuroglial cells. Heparin-Sepharose affinity chromatography of material derived from a human brain homogenate yielded a protein that, when reduced, had an apparent size of 180 kDa and comigrated with reduced platelet thrombospondin on NaDodSO$_4$/PAGE. Immunoblot analysis with monospecific anti-thrombospondin confirmed the presence of immunoreactive thrombospondin. Indirect immunofluorescence of cultured human glial cells indicated the presence of thrombospondin. Metabolic labeling of glial cell cultures with [^{35}S]methionine followed by immunoprecipitation with monospecific anti-thrombospondin revealed synthesis of a 180-kDa polypeptide that comigrated with platelet thrombospondin on NaDodSO$_4$/PAGE. Cultured human glial cells were incubated for 48 hr in serum-free medium with purified platelet-derived growth factor at concentrations up to 50 ng/ml. Aliquots taken at intervals were analyzed by a quantitative double-antibody ELISA. The growth factor stimulated the release of thrombospondin into the culture medium by as much as 10-fold over control cultures. The presence of thrombospondin within glial cells of the central nervous system and the augmentation of its synthesis by platelet-derived growth factor suggest that thrombospondin may play an important role in regulating cell–cell and cell–matrix interactions during periods of cell division and growth.**

Thrombospondin is a 450-kDa glycoprotein (1) with a multinodular structure composed of three 180-kDa subunits (2, 3). Thrombospondin is found in human platelets (4), endothelial cells (5), fibroblasts (6), smooth muscle cells (7), monocytes (8), and granular pneumocytes (9). Thrombospondin has lectin-type activity (10) and interacts in a specific manner with heparin (2), fibrinogen (11), fibronectin (12), collagen (12), histidine-rich-glycoprotein (13), and plasminogen (14). In platelets, thrombospondin is present within the α granule (4, 15) and is expressed on the activated platelet surface upon platelet activation (16). By interacting with fibrinogen, thrombospondin reinforces the strength of interplatelet interactions and plays an important role in determining the size and reversibility of platelet aggregates (17). Thrombospondin forms a ternary complex with plasminogen and tissue plasminogen activator and may serve as a site for localized plasmin generation (14, 18). Endothelial cells, as well as fibroblasts, secrete and incorporate thrombospondin into the extracellular matrix, where it may mediate or modulate cell–substrate and cell–cell interactions (5, 6). Recently, the synthesis of thrombospondin by smooth muscle cells has been reported to be enhanced in the presence of platelet-derived growth factor (PDGF) (19), suggesting that this growth factor may play a role in the expression of this adhesive molecule. In this paper, we present evidence for the synthesis of thrombospondin by astrocytic human glial cells and for the augmentation of this synthesis by PDGF.

MATERIALS AND METHODS

Platelet-Derived Growth Factor. Purified PDGF was a generous gift of T. Deuel (Washington University School of Medicine, St. Louis, MO).

Cells. The human glial cell line AW was prepared in the following manner. Freshly resected tissue removed from a trauma patient was dissected under ×100 magnification to facilitate the removal of the meninges and to cut small pieces of gray matter that were free of large blood vessels. These pieces were minced into a fine pulp and placed in 0.25% collagenase/0.02% DNase (enzymes from Sigma) for 15 min at 37°C in 5% CO_2. The cell suspension was passed through a 100-mesh screen, and the cells were pelleted at 120 × g for 5 min and resuspended in fresh medium. The standard medium used for all cell culture work was Waymouth's MAB 87/3 (Grand Island Biological, Grand Island, NY) containing 25% fetal bovine serum (Biolabs, Logan, UT). No antibiotics were used and the cell line was monitored for mycoplasma by the method of Chen (20).

Two-Dimensional Gel Electrophoresis. Using a modification of the methods of O'Farrell (21), Comings (22), and Garrels (23), total-cell lysates of early-passage cells (serial passage 2) were prepared and analyzed by two-dimensional gel electrophoresis. Briefly, cells in logarithmic growth were grown in medium with [U-^{14}C]leucine (New England Nuclear) for 24 hr and then suspended in lysis buffer (22). Samples were subjected to isoelectric focusing in 19-cm gels cast in 3.2 mm internal diameter Pyrex tubes and run at 800 V for 15 hr and then at 1000 V for an additional 2 hr. The second-dimension electrophoresis (NaDodSO$_4$/PAGE) was performed on a specially constructed apparatus that allows four 20-cm gels to be run simultaneously at 100 V overnight. Analysis of cells for glial fibrillary acidic protein (GFAP) was performed essentially as described (24). After electrophoresis, gels were stained with Coomassie blue R-250, destained, dried, and autoradiographed.

Isolation of Human Brain Thrombospondin. Frozen human brain tissue (158 g), obtained at autopsy, was stripped of adherent membranes, cut into 1-cm pieces, and homogenized

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Abbreviations: PDGF, platelet-derived growth factor; vWF, von Willebrand factor; GFAP, glial fibrillary acidic protein.

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in 280 ml of 136 mM NaCl/25 mM Tris/1 mM EDTA/0.8 mM sodium citrate/1 mM phenylmethylsulfonyl fluoride/50 μM leupeptin at pH 7.4. The homogenate was centrifuged at 100,000 × *g* for 1 hr and a barium citrate precipitate was obtained by the method of Alexander and Detwiler (25). Following washing, a 0.5 M EDTA eluate of the precipitate was obtained in the presence of protease inhibitors and dialyzed overnight against several changes of 200 mM NaCl/55 mM NaOAc/5 mM EDTA/100 μM phenylmethylsulfonyl fluoride at pH 6.0. The dialyzed material was applied to a heparin-Sepharose column (13), and stepwise elution with increasing salt concentration produced a peak at 0.45 M NaCl/10 mM Tris Cl, pH 7.4/2 mM EDTA. $NaDodSO_4$/PAGE was performed (Laemmli system, 3.9% stacking gel, 7.5% separation gel) with ≈8 μg of reduced protein per lane; proteins were stained with Coomassie blue.

Immunoperoxidase Staining of Human Brain Tissue with Monospecific Anti-Thrombospondin. Frozen sections (30-μm) were placed on gelatin-coated glass slides and stained by the peroxidase-antiperoxidase method after incubation with normal serum, anti-thrombospondin, or anti-von Willebrand factor (vWF).

Immunofluorescence Studies of Cultured Human Glial Cells. Normal human glial cells were grown on glass coverslips in Waymouth MAB/20% fetal bovine serum and then were washed in phosphate-buffered saline (0.15 M NaCl/10 mM phosphate, pH 7.4), fixed, and incubated with anti-thrombospondin, anti-vWF, anti-GFAP (Dako, Santa Barbara, CA), or normal rabbit serum prior to incubation with fluorescein-conjugated goat anti-rabbit IgG antibody (Cappel Laboratories, Cochranville, PA) according to methods previously described (26).

Isolation of Thrombospondin from [^{35}S]Methionine-Labeled Human Glial Cells. Normal human glial cells were maintained in culture in Waymouth MAB/20% fetal bovine serum. Near-confluent cultures were metabolically labeled with [^{35}S]methionine for 20 hr. Cell monolayers were solubilized in 3% $NaDodSO_4$ and then made 1.7% (vol/vol) Triton X-100 to form micelles, as described (6). The cell extracts and culture supernatants were preabsorbed with nonimmune rabbit IgG bound to staphylococcal protein A-Sepharose to reduce nonspecific immunoprecipitation. Monospecific anti-thrombospondin (or nonimmune rabbit IgG) bound to staphylococcal protein A-Sepharose was incubated overnight with cell extracts and culture supernatant. After extensive washing in phosphate-buffered saline (pH 7.4) with 0.05% Tween, the Sepharose beads were boiled for 5 min in 2% $NaDodSO_4$ in the presence of 5 mM dithiothreitol and the eluted materials were analyzed by $NaDodSO_4$/7.5% PAGE followed by autoradiography.

PDGF Stimulation of Cultured Human Glial Cells. Normal human glial cells were maintained in Waymouth MAB without serum. After 12 hr, PDGF was added to a final concentration of 5 ng/ml or 50 ng/ml. Aliquots were removed at intervals and assayed for thrombospondin concentration.

Double-Antibody Enzyme-Linked Immunosorbent Assay (ELISA) for Thrombospondin. Control and postculture media were diluted 1:20 and incubated in wells previously coated with a mixture of three anti-thrombospondin monoclonal antibodies developed in our laboratory (Cornell) and used as capturing antibodies. Following washing, the wells were incubated with anti-thrombospondin conjugated to alkaline phosphatase (revealing antibody). Detection with the colorimetric substrate *p*-nitrophenyl phosphate (Sigma) allowed quantitation in a Titertek Multiscan spectrophotometer. Calibration curves were established by using purified platelet thrombospondin.

RESULTS

Isolation of Human Brain Thrombospondin. Frozen human brain tissue stripped of membranes and blood vessels was homogenized in isotonic buffer containing EDTA and protease inhibitors. The homogenate was centrifuged at 100,000 × *g* for 1 hr, and a barium precipitate was obtained from the supernatant (25). The barium precipitate was eluted with 0.5 M EDTA and the eluate applied to a heparin-Sepharose column. At a NaCl concentration of 0.45 M, a protein was eluted that, on reduction and $NaDodSO_4$/PAGE, had an apparent size of 180 kDa and comigrated with purified platelet thrombospondin (Fig. 1). Immunoblotting of the material, using anti-thrombospondin as probe, showed positive reactivity, demonstrating the presence of thrombospondin in the brain homogenate (data not shown).

Immunohistochemical Studies of Human Brain Tissue. To better define the cellular origin of the thrombospondin in our preparation, we performed immunohistochemistry on frozen sections of human brain. Sections (30-μm) were placed on gelatin-coated microscope slides and stained by the peroxidase-antiperoxidase method after incubation with normal serum, anti-thrombospondin, or anti-vWF (Fig. 2). Peroxidase reaction was seen in glial cells in brain sections incubated with monospecific anti-thrombospondin antibody; glial-cell positivity occurred diffusely in cerebral cortex, thalamus, and cerebellum. Controls with nonimmune serum were negative, and sections incubated with anti-vWF showed reaction product only in endothelial cells within blood vessels.

Culture of Normal Human Glial Cells. To examine the possibility of a glial cell origin of the thrombospondin in our preparation, normal human glial cell cultures were prepared from specimens obtained from trauma patients. The cultured cells had typical astrocytic morphology as judged by phase microscopy. Two-dimensional gel electrophoresis was performed to examine the cultures and confirm their glial cell origin. A characteristic pattern of GFAP was observed (22, 24) (Fig. 3), which was blocked when samples were preincubated with anti-GFAP antibody (data not shown). This clearly identifies the cells as astroglial in origin. In addition, immunofluorescence microscopy with anti-GFAP antibody revealed that all cells stained positively (see below), consistent with an astrocytic lineage and excluding contamination by other cell types.

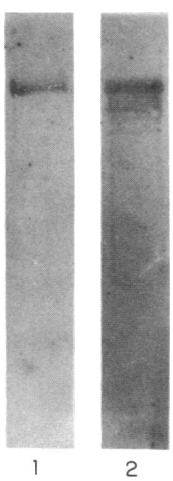

FIG. 1. $NaDodSO_4$/7.5% PAGE of human brain thrombospondin purified by heparin-Sepharose affinity chromatography. Samples (8 μg per lane) were reduced with dithiothreitol; staining was with Coomassie blue. Lane 1: purified platelet thrombospondin, run as standard. Lane 2: heparin-Sepharose eluate.

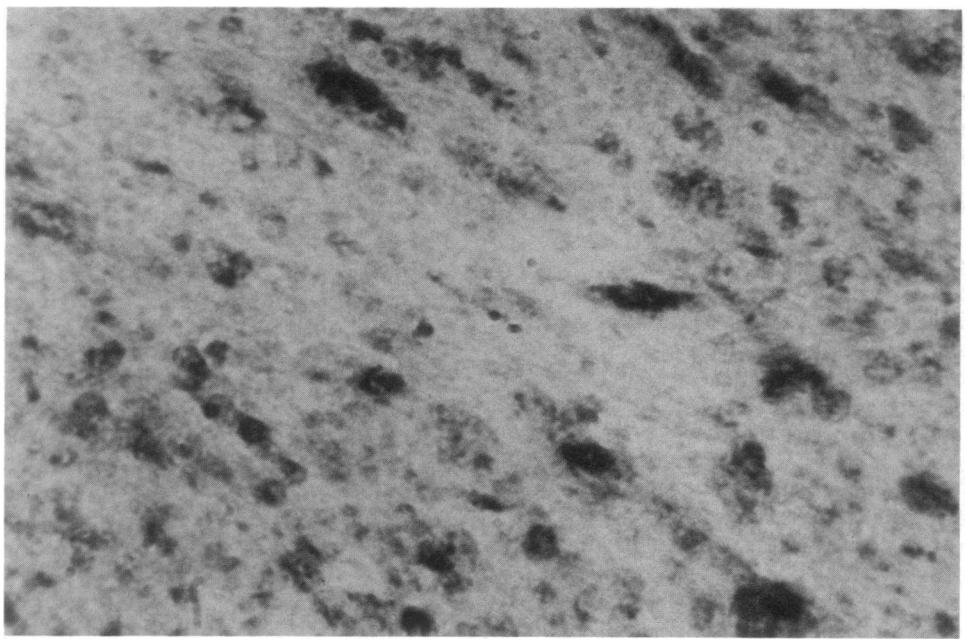

FIG. 2. Immunoperoxidase staining of human cerebral cortex with monospecific anti-thrombospondin. Note reaction product within glial cells. (×640.)

Immunofluorescence Studies of Cultured Human Glial Cells. To confirm the presence of thrombospondin in human glial cells, normal human glial cells grown in cell culture on glass coverslips were incubated with monospecific anti-thrombospondin serum followed by fluorescein-conjugated goat antibody to rabbit IgG. Intense perinuclear fluorescence was observed in all cells (Fig. 4 *a* and *b*). All cells also stained positively with anti-GFAP (a specific marker for glial cells) (Fig. 4 *c* and *d*) and were negative with anti-vWF (a marker for endothelial cells) (Fig. 4 *e* and *f*). The pattern of fluorescence was fibrillar in the cytoplasm, although later-passage cells assumed a less fibrillar perinuclear pattern as has been described by Duffy (27). Since there were no cells that remained unstained by anti-GFAP in these cultures, contamination by fibroblasts is unlikely. Control studies with nonimmune serum were negative.

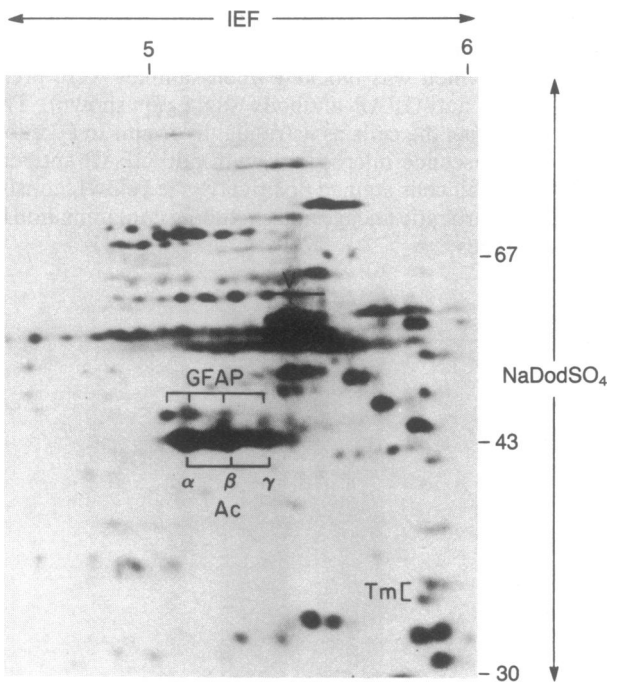

FIG. 3. Two-dimensional gel electrophoresis of cultured human glial cells (line AW). An enlargement of a two-dimensional autoradiogram, depicting the radiolabeled cellular proteins of 30–90 kDa focusing between pH 4.5 and 6.0. Numbers at top indicate the pH gradient established in the isoelectric focusing (IEF) dimension; those at right are molecular mass markers (in kDa) for the second dimension. Several major protein species are identified: α-, β-, and γ-actin (Ac); vimentin (V); tropomyosin (Tm); and GFAP.

Isolation of Thrombospondin from [^{35}S]Methionine-Labeled Glial Cell Cultures. The capacity of cultured normal human glial cells to synthesize and secrete thrombospondin was examined. Glial cells were grown in the presence of [^{35}S]methionine for 20 hr. Thrombospondin was immunoprecipitated from solubilized glial cells and from conditioned culture medium by techniques previously described (6). Analysis of reduced immunoisolates by $NaDodSO_4$/PAGE and autoradiography revealed comigration of the glial cell derived-thrombospondin with purified platelet thrombospondin (Fig. 5).

PDGF Stimulation of Thrombospondin Synthesis by Cultured Human Glial Cells. Purified human PDGF has been reported to stimulate thrombospondin synthesis by cultured smooth muscle cells (19). In order to investigate the regulation of thrombospondin synthesis in human glia, the cells were maintained in serum-free medium in the presence of purified PDGF at 5 or 50 ng/ml. Thrombospondin released by the cells into the culture medium over 48 hr was monitored by double-antibody ELISA (Fig. 6). In both confluent and preconfluent cultures, PDGF stimulated the release of thrombospondin in a dose-dependent manner. Under the serum-free conditions used for these experiments, the concentrations of PDGF added did not result in changes in cell number, and thus the effect of PDGF on thrombospondin synthesis in these cells appears to be independent of its effects on cell proliferation.

DISCUSSION

We have shown that thrombospondin is synthesized by normal human astrocytic glial cells in culture and that this synthesis can be stimulated by PDGF. The presence of

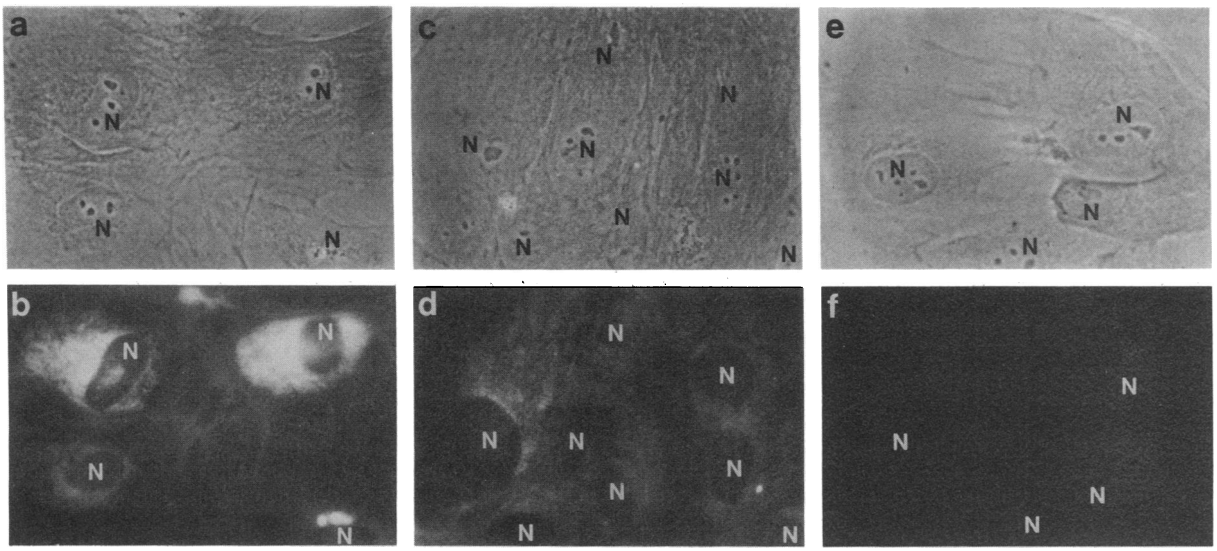

FIG. 4. Immunofluorescence studies of cultured human glial cells. Normal human glial cells were grown on glass coverslips, washed, fixed, and incubated with anti-thrombospondin, anti-vWF, anti-GFAP, or normal rabbit serum prior to incubation with fluorescein-conjugated goat anti-rabbit antibody. Nuclei (N) are identified. (*a*, *c*, and *e*) Phase-contrast micrographs. (*b*, *d*, and *f*) Fluorescence micrographs of the same fields, showing specific perinuclear immunofluorescence of glial cells with anti-thrombospondin (*b*), specific fibrillar cytoplasmic fluorescence of glial cells with anti-GFAP (*d*), and the absence of fluorescence of glial cells with anti-vWF (*f*).

thrombospondin in the brain suggests that it may play a role in the cell-adhesion processes of glial cells during normal development and raises the question of its relationship to the recently described family of cell surface adhesive glycoproteins (29, 32–36). As an extracellular matrix protein, thrombospondin may be a candidate for mediating cell adhesion, as well as modulating a variety of reactions. Thrombospondin binds to plasminogen (14), as well as to histidine-rich glycoprotein (13), and a trimolecular complex of thrombospondin, plasminogen, and histidine-rich glycoprotein has been observed (14). Thrombospondin may therefore regulate plasmin generation. Recently, the plasminogen activator–plasmin system has been implicated in neuronal migration. The migration of neurons from the granular cell layer of the developing rat cerebellum was associated with the extracellular release of plasminogen activator and that migration was inhibited by agents acting on plasminogen activator or plasmin activity (30). Growing glial cells apparently secrete more plasminogen activator than do stable cultures (31), consistent with the idea that plasmin activity plays an important role in cell migration during development. The availability of plasminogen to the cells of the developing embryo has been shown (28).

Cell adhesion is critical to normal development. The migration of cell layers during development involves specific cell–cell and cell–substrate interactions. A family of cell surface glycoproteins that mediate specific cell–cell interactions has been described (29, 32). Specific cell–substrate adhesion molecules that may function in neuromuscular development have also been identified and characterized (33–36). Glial cells provide a scaffolding, upon which neuronal cells can migrate during development, and appear to play a critical role in the repair and regeneration of nervous tissue. During organogenesis of the brain, radial glial cells extend processes that span the entire thickness of the neural tube, and migrating neural cells demonstrate considerable affinity for the glial cell surface during their journey from ependyma to mantle (37). Cultured neural cells preferentially grow on a glial cell substrate (38, 39). In addition, glial cells

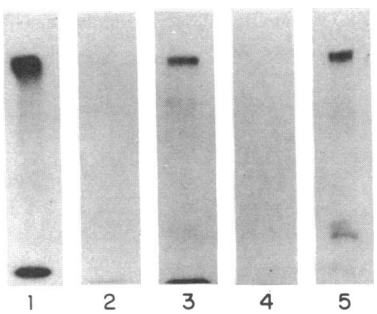

FIG. 5. Autoradiograph of immunologically isolated thrombospondin from [^{35}S]methionine-labeled glial cells. Lane 1: ^{125}I-labeled purified platelet thrombospondin. Lane 2: a control with material immunoprecipitated from glial cell extract with nonimmune rabbit IgG-Sepharose. Lane 3: material immunoprecipitated from glial cell extract with anti-thrombospondin-Sepharose. Lane 4: a control with material immunoprecipitated from culture supernatant with nonimmune rabbit IgG-Sepharose. Lane 5: material immunoprecipitated from culture medium with anti-thrombospondin-Sepharose. The position of the major band in lanes 1, 3, and 5 corresponds to 180 kDa.

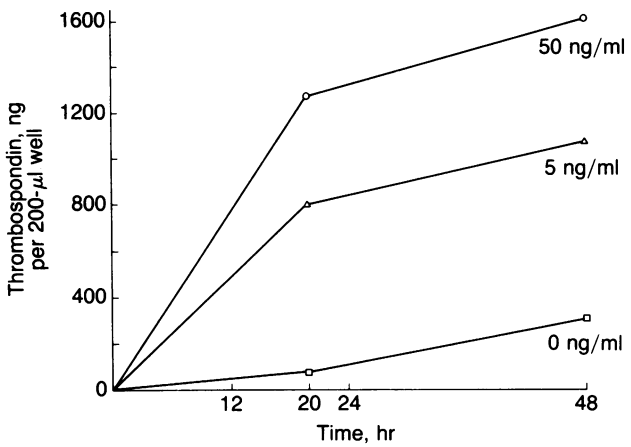

FIG. 6. PDGF-stimulated release of thrombospondin into glial cell culture medium. Glial cells were maintained for 12 hr in serum-free growth medium at a high initial plating density (2×10^5 cells per well). At time zero, purified PDGF (0, 5, or 50 ng/ml) was added; culture medium was withdrawn at intervals and assayed by ELISA.

secrete a protein that, when present on the culture surface, supports neurite outgrowth by neuroblastoma cells in culture (40). A neuronal–glial cell adhesion molecule (Ng-CAM) has been found on neuronal cells; Ng-CAM mediates the interaction between neural cells and glial cells, but it is not expressed during early embryogenesis nor is it found on glial cells (41). More recent data suggest that glial cells express neural cell adhesion molecule (N-CAM) or N-CAM-like molecules in culture (42, 43). The relationship of thrombospondin to this family of adhesive glycoproteins is not known. However, thrombospondin's adhesive properties (10), its ability to bind plasminogen (14) and localize plasmin activity (18), and the modulation of its synthesis by PDGF suggest that it may function as an adhesive or plasminogen-localizing molecule during periods of cell proliferation and growth.

Our results indicate that central nervous system tissue—glia in particular—synthesize thrombospondin, a multifunctional adhesive protein. In addition to its role as an adhesive protein, thrombospondin may be an important modulator of the plasminogen–plasmin system (14, 18) and as such may be critical for normal developmental events. That thrombospondin synthesis in cultured glial cells is stimulated by PDGF seems consistent with expression of thrombospondin by tissues undergoing growth where plasmin-dependent tissue remodeling may be important. PDGF regulation of thrombospondin synthesis has recently been reported in smooth muscle cells (19), suggesting that this may be a general mechanism for thrombospondin expression by other tissues as well.

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