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NEURON LOCALIZATION AND NEUROBLASTOMA CELL EXPRESSION OF BRAIN-DERIVED GROWTH FACTOR

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SUMMARY: Bovine brain-derived growth factor (BDGF) is a ∿16-17 kD polypeptide mitogen with a broad spectrum of cell specificity. Using a highly specific mouse polyclonal anti-BDGF antiserum for indirect immunoperoxidase and immunofluorescent stainings, BDGF was found to be specifically localized in the neurons of bovine brain cortex. The indirect immunofluorescent staining was blocked by the presence of excess purified BDGF. Human neuroblastoma cells showed cytoplasmic staining with anti-BDGF antiserum. The cell lysates of neuroblastoma cells elicited a BDGF-like activity which could be completely inhibited by preincubation with anti-BDGF antiserum.

Bovine brain-derived growth factor (BDGF) is a potent mitogen with a broad spectrum of cell specificity, including endothelial cells, fibroblasts, osteoblasts, astroglial cells, chondrocytes, smooth muscle cells, and epithelial cells (1-3). BDGF appears to be a potent chemotactic factor for fibroblasts and astroglial cells, but not for inflammatory cells such as human peripheral monocytes (4,5). BDGF is a single polypeptide chain of molecular weight ∿16-17 kD whose chemical and physical properties resemble those reported for endothelial cell growth factor (ECGF), brain-derived acidic fibroblast growth factor (acidic FGF), and heparin-binding growth factor α (HBGF-α) (6-11). BDGF differs from basic FGF in chemical, physical, and immunological properties, although both BDGF and basic FGF interact with the same receptor on the cell surface of responsive cells (3,12,13). BDGF receptor has been characterized as a 135 kD protein associated with a protein tyrosine kinase activity (14). The physiological function of BDGF is unknown. The localization of BDGF in brain and its wide spectrum of cell specificity indicate that BDGF may have an important role in the cellular physiology of brain.

In this communication, using an immunoperoxidase staining and immunofluorescent staining with a specific mouse polyclonal anti-BDGF antiserum, we

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describe for the first time the cellular localization of BDGF in neurons of bovine brain cortex. We also show the existence of a BDGF-like molecule in human neuroblastoma cells in terms of growth factor activity and immunoreactivity.

MATERIALS AND METHODS

Materials: Fresh bovine brain was obtained from Schneider Packaging Company, St. Louis, MO. Swiss mouse 3T3 cells, human glioblastoma cells (A-172), and human osteogenic sarcoma cells (HOS) were obtained from the American Type Culture Collection. Rat brain astroglial cells were prepared as described by McCarthy and de Vellis (15) and provided by Dr. Joseph W. Yang, Washington University, St. Louis, MO. The human neuroblastoma cells (esthesio) cell line was established in the Department of Pathology, St. Louis University School of Medicine, from a frontal lobe and cribiform plate mass occurring in a 46 year-old female Caucasian. Rabbit anti-human neuron specific enolase, biotinylated goat anti-mouse immunoglobulins, avidin-labeled peroxidase, and streptavidin-phycoerythrin were obtained from Biogenex, CA. Fluorescein-labeled $F(ab)_2'$ goat anti-rabbit immunoglobulins were obtained from Accurate Chemical and Scientific Company, NY. BDGF (mol wt ∿16,000) and mouse polyclonal anti-BDGF antiserum were prepared as previously described (3). This polyclonal anti-BDGF antibody reacted with BDGF, but not with basic fibroblast growth factor (FGF) in the Western blot analysis (3). Bovine pituitary basic FGF was obtained from Collaborative Research and used without further purification. [Methyl-^{3}H]thymidine (79.4 Ci/mmol) was purchased from New England Nuclear.

Immunoperoxidase Staining: Fresh snap frozen tissues of frontal lobe and paratal lobe of bovine brain were cut into ∿5-6 μ cryostat sections, dried in air and fixed in 100% acetone. The fixed tissues were incubated with mouse anti-BDGF antiserum (1:100 dilution), followed by sequential incubation with biotinylated goat anti-mouse IgG, avidin-labeled peroxidase and peroxidase substrate solution (16).

Immunofluorescent Staining: The fixed tissue slides were incubated with mouse anti-BDGF antiserum (100 x), followed by incubation with fluorescein-labeled $F(ab)_2'$ rabbit anti-mouse IgG (17).

Double Immunofluorescent Staining: Fixed cultured cells were incubated with mouse anti-BDGF antiserum (1:100 dilution) and rabbit anti-neuron specific enolase (1:500 dilution), followed by incubation with fluorescein-labeled goat anti-rabbit IgG and biotinylated goat anti-mouse IgG, plus streptavidin-phycoerythrin. The stained slides were viewed and photographed with a Leitz orthoplan fluorescent microscope with filters, 520 nm and 566 nm for green and red fluorescent, respectively.

RESULTS

To investigate the physiological role of BDGF, we raised a mouse anti-BDGF antiserum, demonstrated its specificity (3), and used it for immunolocalization of BDGF in brain. When bovine cerebral cortex tissue slides were stained with Meyer's hematoxylin solution, followed by sequential incubation with anti-BDGF antiserum, biotinylated goat anti-mouse IgG, avidin-labeled peroxidase and peroxidase substrate solution (16), BDGF was revealed exclusively in the neurons (Figure 1). When bovine cerebral cortex tissue slides were stained with anti-BDGF antiserum and fluorescein-labeled $F(ab)'_2$ rabbit anti-mouse IgG (17), neurons in gray matter (Figure 2A, right) and

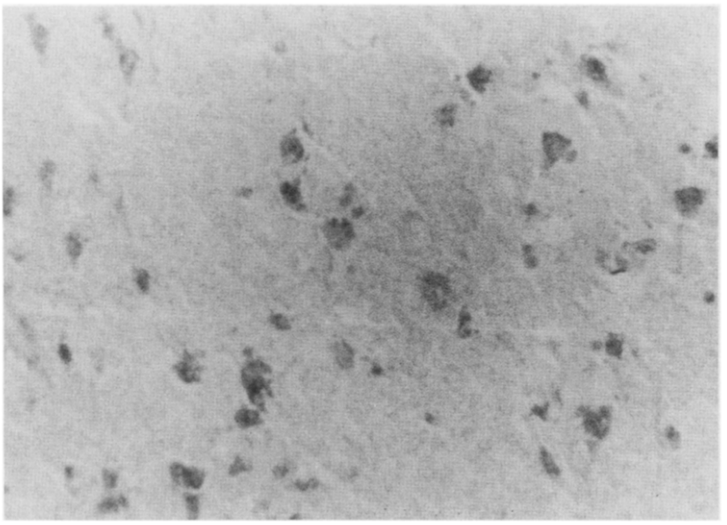
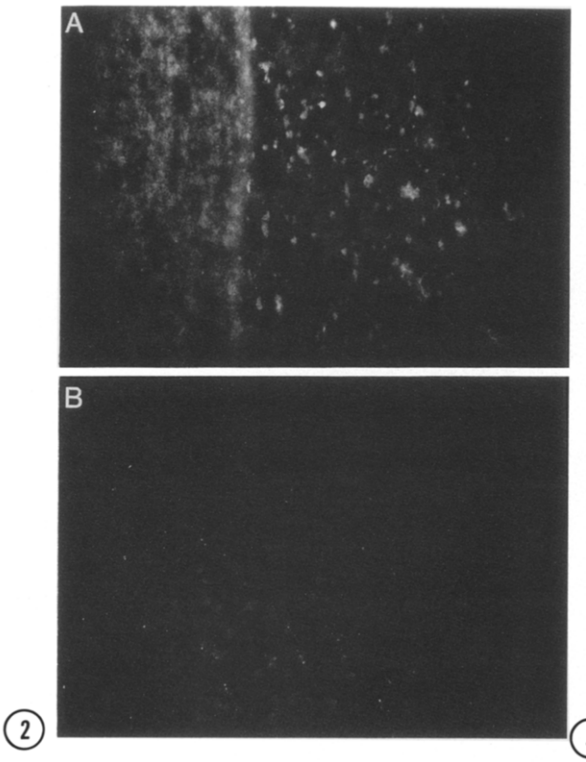


Figure 1. Immunoperoxidase staining of bovine cerebral cortex with a mouse anti-BDGF antiserum. After staining with Meyer's hematoxylin solution, the tissue slides were incubated with mouse anti-BDGF antiserum (1:100 dilution), followed by sequential incubation with biotinylated goat anti-mouse IgG, avidin-labeled peroxidase and peroxidase substrate solution (3-amino-9-ethyl carbazole-N,N'-dimethyl formamide and H_2O_2). The cells stained by the immunoperoxidase technique (dark color) were identified as neurons by histomorphology. Magnification: 150X.

axons in the white matter (Figure 2A, left) of cortex showed positive fluorescence. The immunofluorescent staining of neurons with anti-BDGF antiserum can be completely blocked by the presence of BDGF (1 μg/ml) (Figure 2B), suggesting that the immunofluorescent staining is due to a specific interaction between anti-BDGF antibody and BDGF in neurons. Using this indirect immunoflourescent staining procedure, we have also examined other tissues and cultured cells for the presence of BDGF. These include human neuroblastoma cells, osteogenic sarcoma cells, prostatic carcinoma cells, and glioblastoma cells. Specific markers for each cell type have also been examined with appropriate specific antibodies. Human neuroblastoma cells also showed staining with anti-BDGF antiserum, whereas the other cell types tested did not. In human neuroblastoma cells, several cytoplasmic staining patterns were observed with anti-BDGF antiserum. These include perinuclear submembrane localization and accentuation in the axon dendrites pods (Figure 3A). These neuroblastoma cells were also characterized by immunofluorescent staining with anti-human neuron specific enolase (Figure 3B). To investigate the biological activity of BDGF or BDGF-like antigen in these tumor cells of neurons, the mitogenic activity of cell lysates of neuroblastoma cells were assayed with Swiss mouse 3T3 cells. The cell lysates of neuroblastoma cells elicited a very potent mitogenic response in 3T3 cells. Most of the mitogenic activity of cell lysates was retained by heparin-Sepharose gels and eluted with 2 M



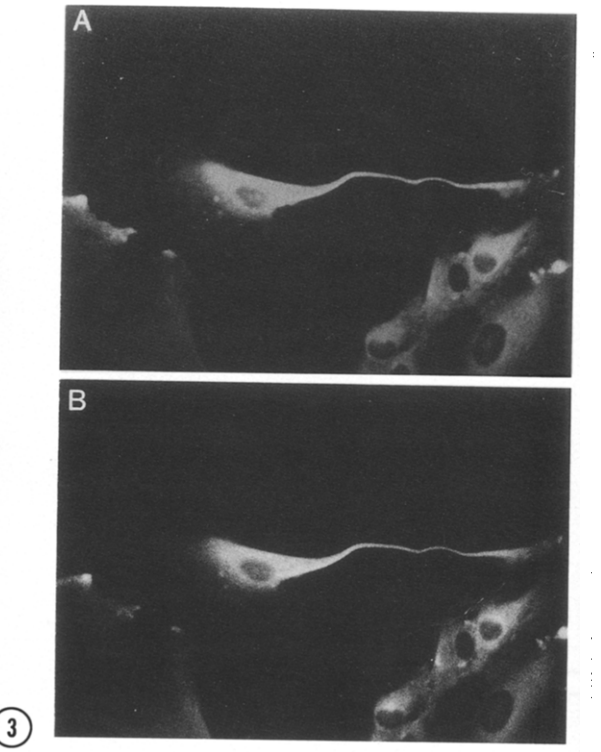


Figure 2. Immunofluorescent staining of bovine cerebral cortex with a mouse anti-BDGF antiserum. The tissue slides were fixed in 100% acetone and incubated with mouse anti-BDGF antiserum (1:100 dilution) in the absence (A) and presence (B) of 1 μg/ml of BDGF, followed by incubation with fluorescein-labeled $F(ab)'_2$ rabbit anti-mouse IgG. The neurons in gray matter (A, right) and axons in the white matter (A, left) showed positive fluorescence. Magnification: 96X.

Figure 3. Double immunofluorescent staining of human neuroblastoma cells with a mouse anti-BDGF antiserum and a rabbit anti-human neuron specific enolase antiserum. Human neuroblastoma cells (esthesio) were grown on tissue culture chamber/slides in DMEM containing 10% fetal calf serum, fixed in 80% acetone and incubated with mouse anti-BDGF antiserum (1:100 dilution) and rabbit anti-neuron specific enolase (1:500 dilution), followed by incubation with fluorescein-labeled goat anti-rabbit IgG and biotinylated goat anti-mouse IgG, plus streptavidin-phycoerythrin. (A) shows the immunofluorescent staining with anti-BDGF antiserum; (B) shows the immunofluorescent staining with anti-neuron specific enolase antiserum. Magnification: 300X.

NaCl solution (Table I). The 2 M NaCl eluent stimulated the DNA synthesis of Swiss mouse 3T3 cells in a dose dependent manner (Figure 4). This high affinity binding of the mitogenic activity to heparin-Sepharose gels and the dose response profile in stimulation of DNA synthesis of 3T3 cells are very similar to those of BDGF (3,13). Furthermore, the mitogenic activity of the 2 M NaCl eluent could be completely inhibited by preincubation with anti-BDGF antiserum, whereas the preimmune serum did not have effect on the mitogenic activity of the cell lysates of neuroblastoma cells (Figure 4). These results suggest the expression of BDGF or a BDGF-like molecule in neuroblastoma cells.

TABLE I

Heparin-Sepharose Gel Fractionation of the Mitogenic Activity in the Cell Lysates of Human Neuroblastoma Cell

	Fraction	[Methyl-^{3}H]thymidine incorporation into Swiss mouse 3T3 cell DNA
		cpm/well
1.	No addition	1510 ± 165
2.	Cell lysates before heparin absorption	7725 ± 310
3.	Unretained fraction	1625 ± 195
4.	1 M NaCl eluent	2130 ± 145
5.	2 M NaCl eluent	6060 ± 220

Human neuroblastoma (esthesio) cells were grown to confluence in 7 T-75 flasks in DMEM containing 10% fetal calf serum. The cells were washed three times with phosphate buffer saline, pH 7.4, and then harvested by scraping. The cell pellets were then suspended in 1 ml of 5 mM HEPES buffer, pH 7.4, frozen and thawed and homogenized with a Dounce homogenizer. After centrifugation, the supernate (cell lysates) was mixed with 0.5 ml of heparin-Sepharose gel. After 3 h at 4°C, the gel suspension was centrifuged, and the supernate (the unretained fraction) was collected. The gel was then suspended in 1 ml of the same buffer containing 1 M NaCl. After 2 h mixing at 4°C, the gel was centrifuged, and the supernate (1 M NaCl eluent) was collected. The gel was resuspended in 1 ml of the same buffer containing 2 M NaCl. After mixing at 4°C for 2 h, the gel suspension was centrifuged, and the supernate (2 M NaCl eluent) was collected. Ten µl of each fraction collected as described above was assayed for growth factor-dependent [methyl-^{3}H]thymidine incorporation into Swiss mouse 3T3 cell DNA (3).

DISCUSSION

The specific localization of BDGF in neurons indicates a potential role of BDGF in the physiological function of neurons. With the techniques used, we could not be certain whether the BDGF is produced in neurons or is produced in other cells and taken up by neurons. Its presence in neuroblastoma cells in cell culture seems to indicate that BDGF is produced in this cell type. However, the expression of the growth factor in these malignant neuroblastoma cells may be different from that in the normal counterpart.

BDGF has been shown to be mitogenic for astroglial cells (2,3,13). Thus, if produced and secreted by neurons, BDGF could be very important for growth of astroglial cells and possibly oligodendrocytes in development. Recent observations (18,19) have shown that neurons provide molecules that stimulate the growth of oligodendrocytes. Waliche and his co-workers have recently reported that basic FGF promotes neuronal survival and neurite extension (20). Since BDGF and basic FGF interact with the same receptor on the cell surface of several types of responsive cells (3), it is possible that BDGF elicits a

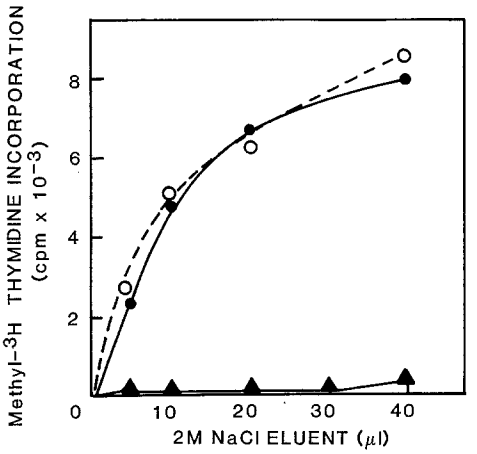


Figure 4. Concentration dependence of the 2 M NaCl eluent from heparin-Sepharose gel fractionation of neuroblastoma cell lysates in stimulation of DNA synthesis in Swiss mouse 3T3 cells. The 2 M NaCl eluent from heparin-Sepharose gel fractionation of human neuroblastoma (esthesio) cell lysates was obtained as described in the footnote of Table I. Monolayers of Swiss mouse 3T3 cells were incubated with different amounts of the 2 M NaCl eluent from heparin-Sepharose gel fractionation in 0.5 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 5% human plasma-derived serum and [methyl-^{3}H] thymidine (0.3 μCi/μg/ml). After 20 h at 37°C, the mitogenic activity assay was stopped. In a parallel experiment, the various amounts of the 2 M NaCl eluent were preincubated with 10 μl of anti-BDGF antiserum (100X dilution) or preimmune serum (100X dilution). After 4 h at 37°C, the reaction mixture was centrifuged at 12,000 x g and the supernates were then added into the assay medium. ● - ●, no pretreatment; ○ - ○ pretreatment with preimmune serum; ▲—▲ pretreatment with anti-BDGF antiserum.

similar neuronal response. If so, an autocrine role of BDGF in neuronal growth during normal development of the central and peripheral nervous systems would be plausible.

Many growth factors have been isolated from neural tissues, including brain, hypothalamus and retina (1-3,6-11,21,22). The cellular origins of these growth factors have not been reported. BDGF is the first of these to be localized to a specific cell type. It seems likely that BDGF is in fact neuron-derived, as well as neuron-associated. The antibody used in these localization studies should be useful in defining the site of synthesis and the route of secretion of this growth factor in neurons and in neuron-related disease.

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