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CLINICAL STUDY

Problematic visualization of human protoplasmic astrocytes Immunohistochemical stains

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ABSTRACT
OBJECTIVE: Traditionally, astrocytes are categorized into fibrous and protoplasmic types based on their morphological appearance.
BACKGROUND: For a long time, glial fibrillary acidic protein (GFAP) has been considered the best astroglial marker. However, protoplasmic astrocytes stain negatively for GFAP using immunohistochemical methods.
METHODS: Immunohistochemical methods with antibodies to GFAP were used to identify astrocytes. Paraffin sections were prepared from brain biopsy samples of adult patients diagnosed with aneurysm, traumatic contusion, subdural hematoma, gliomas or brain metastases.
RESULTS: In all samples, the GFAP-positive fibrous astrocytes were located in the subpial area and in the white matter. Several GFAP-positive protoplasmic astrocytes were found only in one brain sample from a patient with ruptured aneurysm. Conversely, GFAP-positive astrocytes of intermediate shape were rarely observed in the cortical gray matter from patients with tumoral diagnoses.
CONCLUSION: Our immunohistochemical study demonstrates that GFAP-positive cells with morphology similar to protoplasmic astrocytes rarely occur in injured brain cortex. We conclude that brain tissue contains GFAP-negative glial precursor cells, which can differentiate into GFAP-positive cells under pathological conditions and sometimes exhibit protoplasmic or intermediate morphology. Similarly, GFAP staining is increased in fibrous astrocytes, typically described as reactive to brain noxa. These results raise many questions about astrocytes identification and classification. In addition, these findings may explain the absence of GFAP-positive cells in adult human brain cultures, often termed "glia-like" cells *(Fig. 3, Ref. 18)*. Text in PDF www.elis.sk
KEY WORDS: protoplasmic astrocytes, fibrous astrocytes, GFAP, human glia, immunohistochemistry.

Introduction

Virchow (1846) coined the term *Nervenkitt* or nerve-cement, later translated as neuroglia, which he considered as connective tissue, or "nerve" glue containing cellular elements. Michael von Lenhossek (1893) introduced the term astrocytes for star-shaped neuroglial cells, and Kolliker and Andriezen categorized astrocytes into fibrous and protoplasmic types (1). Other authors reported that distinction between gray matter protoplasmic and white matter fibrous glia was made by Andriesen (1893). Ramon y Cajal adopted this classification but changed the name glia into astrocytes. He introduced his own gold chloride sublimate method (1913). This method enabled him to recognize that fibrils were present in astrocytes of both gray and white matter (2,3,4). The structure of gliofibrils become clear by isolation of GFAP(5). Later studies showed that gliofibrils are intermediate filaments, GFAP together with vimentin. Fibrous astrocytes, characterized by straight long and branching processes, are mainly located in the white matter, whereas protoplasmic astrocytes with highly branched processes are distributed in the gray matter.

Recently a new astrocyte classification proposed by Raff and his colleagues (7), distinguishing type 1 (GFAP+/A2B5-) and type 2 (GFAP+/A2B5+) of astrocytes, analogous to protoplasmic and fibrous astrocytes, respectively (8). Previously we reported that A2B5 antigen, described as a complex ganglioside found in plasma membranes of retinal neuron cell bodies (9), was expressed only in a small subset of GFAP+/A2B5+ in adult human brain cultures (10). Consistent with our (A2B5) data, the study of Newcombe (11) revealed that approximately 10% of astrocytes with long processes were A2B5-positive. However, A2B5staining was not detected *in situ* in the nervous systems of vertebrates (2), suggesting its expression may be induced under culture conditions in a specific subpopulation of GFAP-positive human astrocytes.

In this study, we aim to address basic questions regarding protoplasmic astrocytes in adult human brain cortex, including their GFAP expression in both tumoral and non-tumoral brain samples.

Materials and methods

Brain biopsy materials

Brain samples were kindly provided by the Department of Neurosurgery, Derer's Hospital, Bratislava. This study reports

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on findings from brain biopsies collected between 1989 to 2000. Experiments involving human brain biopsies were conducted in accordance with Slovak laws 272/1994, 76/2004 and were approved by the Ethical Committee of UNB Bratislava. For this study we selected brain biopsies from adult patients undergoing neurosurgical intervention for ruptured aneurysm (n=2), traumatic contusion (n=1), subdural hematoma (1), gliomas (n=6) and brain metastases (n=2). The samples were obtained from the temporal or frontal lobes. The brain samples were fixed in 4% neutral formalin and embedded in paraffin for immunohistochemistry.

Immunohistochemical stains

To detect astroglial cell types, we used antibodies against GFAP: clone GF-01 (1:100, Exbio, Prague), clone G-A-5 (1:100, Sigma), and polyclonal sera to GFAP (1:100, Dako). Formalin-fixed, paraffin-embedded sections were used for indirect immunoperoxidase or PAP staining. The diaminobenzidine reagent was used as the chromogen and the sections were stained with hematoxylin for 30 s.

Results

Immunohistochemical staining for GFAP

For astrocytes detection, we used indirect immunoperoxidase staining or PAP method, employing two monoclonal antibodies and polyclonal sera against GFAP. Both methods and all antibodies yielded similar staining results, although staining intensities varied, reflecting mainly the hypertrophy of reactive astrocytes. In all brain sections, GFAP-positive staining was observed in the subpial area (layers I–II) and in the subcortical white matter; these cells were identified as fibrous astrocytes. Conversely, morphologically distinct protoplasmic astrocytes were observed in only one brain biopsy sample. Surprisingly, GFAP staining varied in brain biopsies from patients of similar ages and diagnosis, specifically those with brain hematoma caused by ruptured aneurysm.

Case A: Brain sections of the frontal lobe obtained from a 34-year-old woman (Fig. 1A–I) showed positive staining for GFAP in the subpial area (Fig. 1A) and in subcortical white matter

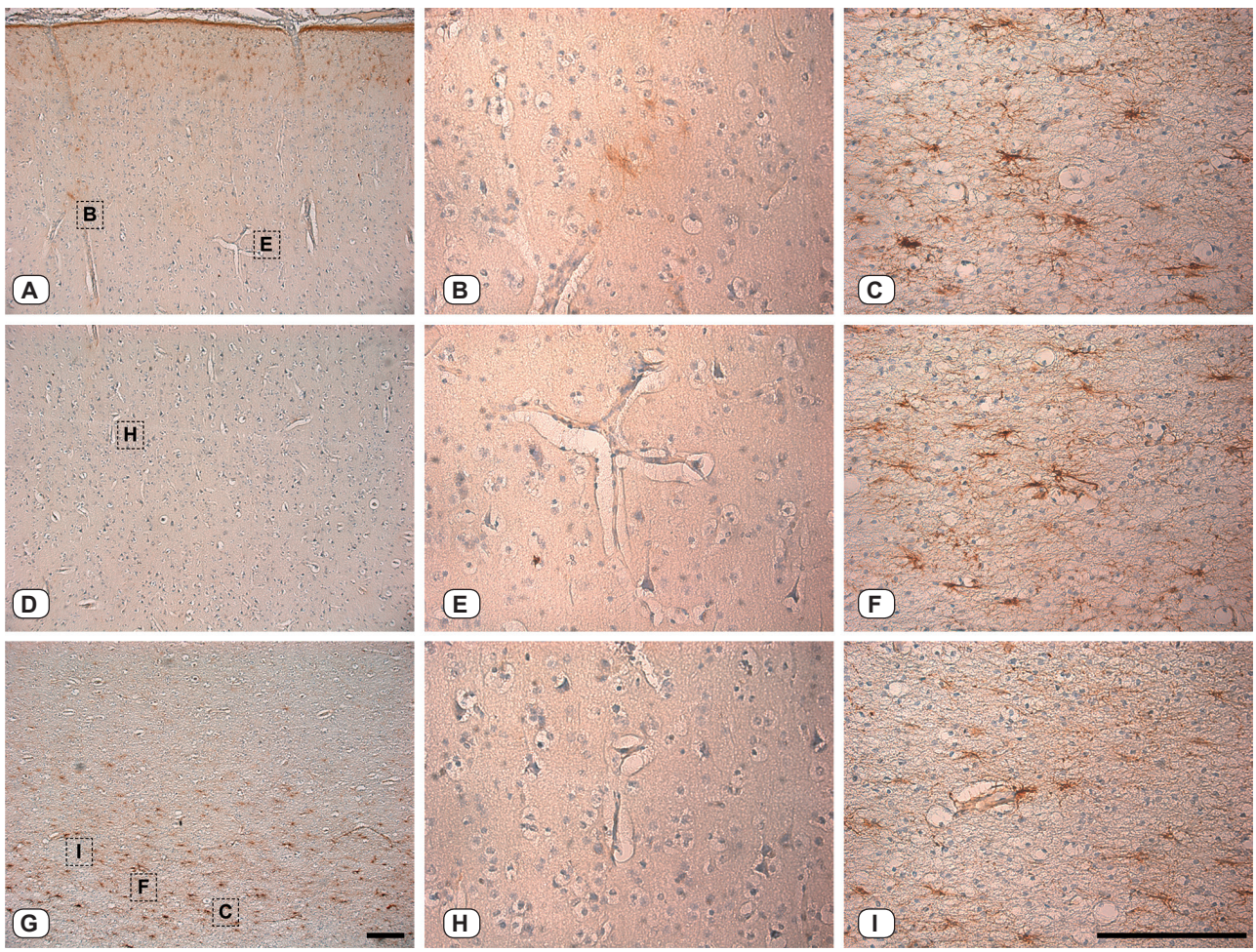

Fig. 1. Immunohistochemical GFAP staining of brain tissue from a 34-year-old woman with hematoma and aneurysm. Positive staining of fibrous astrocytes in the subpial area (A) and subcortical white matter (G). Absence of positive protoplasmic astrocytes in the brain cortex (A, D, G), details (B, E, H). Morphological features of fibrous astrocytes (C, F, I). Scale bars=50 um.

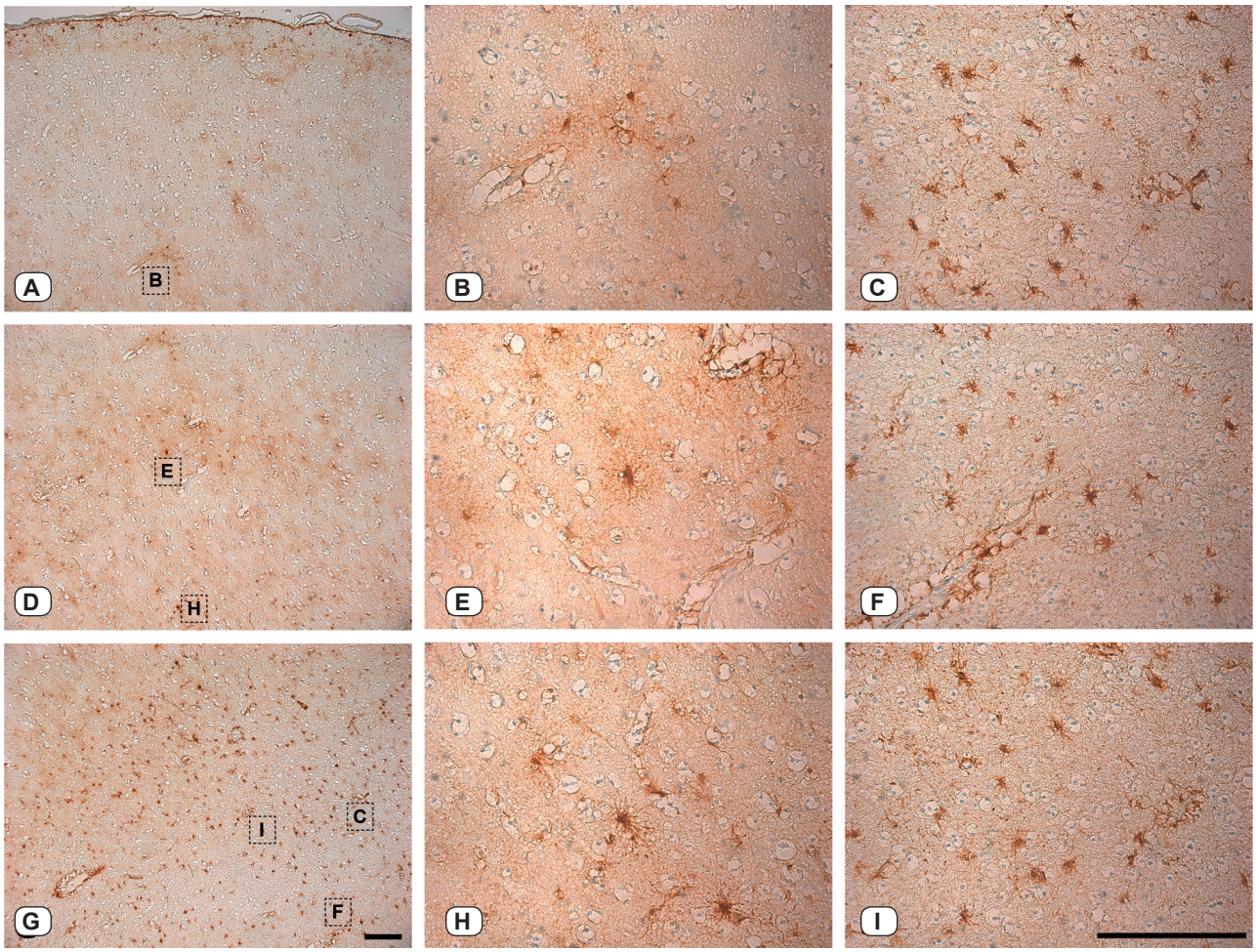

Fig. 2. Immunohistochemical GFAP staining of brain tissue from a 42-year-old woman with hematoma and aneurysm. Positive staining of fibrous astrocytes in the subpial area (A) and subcortical white matter (G). Positive astrocytes in the brain cortex (A, D), details showing protoplasmic and intermediate astrocyte morphology (B, E, H). Morphological features of fibrous astrocytes (C, F, I). Scale bars=50 um.

Fig. 1C, F, I. The brain cortex (layers III–VI) was GFAP-negative (Fig. 1D, E, F).

Case B: Brain sections of the frontal lobe obtained from a 42-year-old woman (Fig. 2A–I) showed positive staining for GFAP in the subpial area and subcortical white matter (Fig. 1A, C, F, I). However, this sample was the only one with several GFAP-positive cells similar to protoplasmic astrocytes observed in the brain cortex (layer III–VI) (Fig. 2A, B, D, E, H). In two other samples from patients with brain contusion and subdural hematoma, the staining was similar to that described in case A.

Staining of brain sections from patients with brain metastasis (Fig. 3A, B) or brain gliomas (Fig. 3C–E) showed GFAP results similar to those described in case A. However, in the brain cortex, the staining revealed small numbers of GFAP-positive cells, found mainly as single cells or sometimes as small groups of cells with intermediate astroglial morphology, but none with a clearly protoplasmic shape (Fig. 3A–F). Fibrous astrocytes in the white matter were strongly stained for GFAP (Fig. 3A, F), and exhibited features of reactive astrocytes (Fig. 3A, E)

Discussion

Previously, we addressed the question of rare appearance of GFAP-positive cells in adult human brain cultures (12, 13). The presence of astrocytes was examined by immunohistochemical methods in adult human brain tissue from patients with non-malignant diagnoses. Unexpectedly GFAP-positive cells were only observed in the subpial area and in the white matter. No GFAP-positive cells were detected in the cortical gray matter, (layer III-VI). A similar localization of GFAP-positive cells was observed in rat brain, visualized by *in situ* hybridization (14). In normal brain, numerous astrocytes were described as GFAP-negative, predominantly in the gray matter (2). The problem of astrocyte identity or the crisis of cell identification has been addressed in several studies (4, 15)

In this study, we examined brain biopsy samples from patients with trauma, aneurysm, gliomas or brain metastases, aiming to visualize distinct protoplasmic astrocytes using GFAP immunohistochemistry. GFAP-positive staining was consistently

observed in the white matter and subpial area (layer I–II) across all brain sections, morphologically defining these cells as fibrous astrocytes. Unexpectedly, GFAP-positive cells were found in the brain cortex (layer III–VI) in only one brain sample with aneurysm. These astrocytes displayed intermediate morphologies, some resembling protoplasmic astrocytes. However, in another sample from a patient of similar age and identical diagnosis, the GFAP-positive cells were absent in the brain cortex. Conversely, GFAP-positive cells were observed in the brain cortex of patients diagnosed with brain gliomas or metastases, albeit rarely and mainly as single cells with various intermediate morphologies.

Based on our previous *in vitro* studies, we concluded that GFAP-negative glial precursors are scattered throughout the entire adult human brain. Cultures derived from adult human brain consist predominantly of GFAP-negative flat cells. Only a small number of GFAP-positive cells persist in culture derived from white matter in early passages. However, as cells' growth slows down spontaneously, there is an induction of GFAP expression and morphological differentiation of initially GFAP-negative cells. Cells in non-proliferating cultures at higher terminal passages displayed high morphological heterogeneity in size and shape. However, in no cases, we observed a protoplasmic morphology of astrocytes with induced GFAP expression.

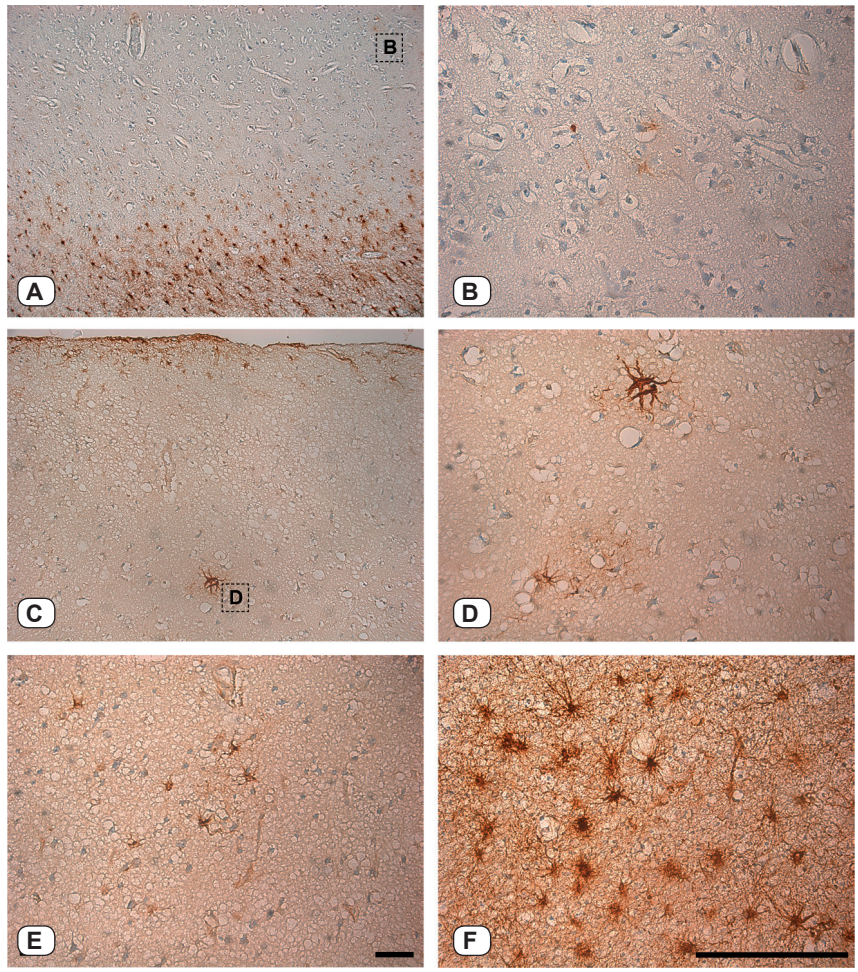

Fig. 3. I. Immunohistochemical GFAP staining of brain tissue from a 59-year-old male with brain metastasis (A-B), and a 60-year-old male with glioma (C-F). Positive staining in the subcortical white matter (A) and subpial area (C). Brain cortex with astrocytes of intermediate morphology (C, E), details (B, D). Noteworthy findings of reactive fibrous astrocytes (A, F) and absence of protoplasmic astrocytes in the brain cortex. Scale bars=50 um.

Similar processes may be observed in the brain tissue under pathological conditions, typically accompanied by varying stages of intracranial pressure. It is generally acknowledged that astrocytes react to brain noxa, exhibiting hypertrophy and increased GFAP staining intensity. However, our study demonstrates that only fibrous astrocytes become reactive under pathological conditions, such as following prolonged periods of chronic intracranial pressure. Conversely, GFAP-negative precursor cells in the cortical gray matter may differentiate into GFAP-positive protoplasmic astrocytes.

These findings may reflect unknown processes of astrocytes differentiation during short periods of intensified intracranial pressure (as in aneurysm) or prolonged chronic brain pressure (as in brain tumors). It is problematic to determine the extent of injury required to induce differentiation into protoplasmic astrocytes, particularly in cases involving high intracranial pressure.

In this study we present controversial results regarding GFAP immunohistochemical staining of human astrocytes in cortical gray and white matter. While fibrous astrocytes show intense GFAP staining, it remains unclear why protoplasmic astrocytes stain negatively on paraffin sections. This discrepancy might be partially explained by alterations in GFAP epitopes due to tissue fixation (16, 17, 18). However, the mechanism behind their transition to positivity in injured brain tissue remains unresolved. These results raise several questions about astrocyte classification, impregnation techniques in comparison with immunohistochemical methods, and primarily about astrocyte functions which we do not know clearly visualized.

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