Journal of Supramolecular Structure 11:269–281 (1979)
Tumor Cell Surfaces and Malignancy 215–227

Fibronectin Associated With the Glial Component of Embryonic Brain Cell Cultures

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In a basic approach to investigations of neuronal–glial interactions during both normal brain development and its pathogenesis, embryonic brain cell populations were fractionated into purified neuronal and glial components. Using separation procedures based on differential adhesion and cytotoxicity, the isolated neuronal and glial phenotypes could be identified by distinct morphological and biochemical characteristics, including the visualization of glial fibrillary acid protein (GFA) within glial cells in immunohistochemical assays with monospecific anti-GFA serum.

When unfractionated cerebrum cells dissociated from 10-day chick or 14-day mouse embryos were plated as monolayers and cultured for 1–14 days, monospecific antiserum against fibronectin (LETS glycoprotein) was found to react with many, but not all, of the cells as revealed by indirect immunofluorescence microscopy. The isolated neuronal and glial components of these populations were used to determine whether the appearance of membrane-associated fibronectin was characteristic of one cell type or the other, or both, and if neuronal–glial cell interaction was required for its expression. It was found that the surfaces of glial cells, completely isolated from neurons, showed an intense fluorescent reaction to the anti-fibronectin serum. In contrast, the purified neuronal cultures showed no fluorescence with either the anti-GFA or anti-fibronectin sera. These results demonstrate fibronectin as a cell surface protein associated primarily with glial cells and independent of neuronal–glial cell interaction for its expression. Furthermore, the results indicate that the fibronectin observed on glial cell surfaces in these cultures is produced endogenously and is not due to the preferential binding of fibronectin present in the culture medium. The role of fibronectin as an adhesive molecule in neuronal–glial interactions is discussed.

Key words: **fibronectin, cell fractionation, glial fibrillary acidic protein, immunofluorescent labeling, neuronal–glial cell interactions, brain cell culture.**

The role of the cell surface in mediating many important cellular functions such as intercellular adhesion [1], motility [2], growth control [3], and selective transport [4] has stimulated widespread interest in identifying function-related membrane macromolecules both in normal and in pathological tissues, with special emphasis on neoplasia [5–7].

Received May 3, 1979; accepted July 16, 1979.

0091-7419/79/1102-0269$02.60

Although such studies have been useful in characterizing membrane constitutents of a wide variety of cell types, progress concerning cells derived from the central nervous system has been hampered by the inherent complexities encountered when working with brain tissues, particularly with respect to the heterogeneity of cell types and the paucity of reliable biochemical markers for their identification. Despite these difficulties, a 54,000 dalton polypeptide originally obtained in purified form from multiple sclerosis plaques of human brain tissue [8,9], and more recently from normal human brain [10], has been revealed to be expressed exclusively by neuroglia, particularly the astrocytic components [9], and to be correlated with cytoplasmic 100 Å filaments [11]. Due to an abundance of dicarboxylic amino acids this protein has been termed the glial fibrillary acidic protein (GFA) and is used as a diagnostic indicator for glial cells.

Recently, much attention has been focused on a high molecular weight (220,000 dalton) glycoprotein that has been shown to be a prominent cell surface component of many cell types grown in vitro, including fibroblasts [12–16], epithelial cells [17], myoblasts [18–20], and vascular endothelial cells [21–23]. Furthermore, this substance, now referred to as fibronectin, has been demonstrated to be a constitutent of plasma and serum, present in high concentration, and antigenically identical to the cold-insoluble globulin (CIg) reported by Mosseson [24–27]. The ubiquitous nature of fibronectin exhibited in vitro reflects its presence in a wide variety of embryonic and adult tissues examined in situ [28–30], where it is found primarily in association with basement membrane and connective tissue [31,32]. Examination of cells following neoplastic transformation reveals a marked reduction or complete absence of fibronectin [33–36]. This loss of fibronectin is a result of decreased biosynthetic activity [37] and has been correlated with the tumorigenic capabilities of cells in a series of adenovirus-transformed rat cell lines [38]. Fibronectin has been postulated to play a role in cell adhesion [39,40] and to influence cell morphology [41–43] and motility [44]. If such is the case, then expression of this glycoprotein by cellular elements of the brain during neurogenesis may be of importance in understanding the many complex cell migrations and interactions that occur during development of the central nervous system and in elucidating the alterations in cell behavior associated with neoplasia.

To approach the question of cell surface fibronectin expression in the developing brain, we have examined monolayer cultures of embryonic cerebral cells by indirect immunofluorescent assays using antisera directed against purified fibronectin. The use of methodologies developed in this laboratory for the preparation of homogeneous populations of neurons or glia suitable for extended culture and histochemical analysis permitted the localization of fibronectin with respect to particular cell types. We have obtained evidence, reported here, that developing glial cells, whose identity is verified by immunofluorescence assays with anti-GFA serum, produce cell-surface-associated fibronectin. In contrast, fibronectin is not detectable in pure neuronal cultures. The implications of glial fibronectin for neuronal adhesion and migration during normal development and also in neoplasia are considered in the discussion.

METHODS

Preparation of Cell Cultures

Using sterile techniques, cerebral lobes from 10-day chick or 14-day mouse embryos were dissociated into single cell suspensions according to standard procedures developed by Garber and Moscona [45]. Embryos were staged using previously established guidelines [46,47]. The tissues were freed of meninges, cut into 2mm^3 pieces, incubated in calcium-

magnesium-free Tyrode's solution (CMF) at 37°C for 20 min, and then enzymatically dissociated in 0.1% trypsin (Grand Island Biological) at 37°C for 35–40 min under a 5% CO_2–95% air atmosphere. After 3 washes in CMF, culture medium was added and the tissues were dispersed into single cell suspensions by flushing them repeatedly through a fine-bore pipette. Aliquots ($3–6 \times 10^6$ cells/dish) were dispensed into 35 mm Falcon tissue culture dishes containing 3 ml of culture medium (Eagle's basal medium, supplemented with 3.8 mg/ml d-glucose, 15% fetal bovine serum, 1% l-glutamine, and 1% penicillin-streptomycin solution, 50 units each/ml).

Homogeneous glial cultures were prepared by taking advantage of the ability of glial cells to adhere to the underlying substratum at a faster rate than neurons [48]. Selected brain cell suspensions were plated for 10 min, then the supernatant medium containing the unattached neurons was removed, and fresh medium was added. A glial-enriched culture was obtained from the plated cells. Residual neuronal contaminants were effectively removed by subculturing the glial cultures after they reached confluence. The neurons' restricted capacity to reattach in secondary culture, plus the glial cells' continued mitotic activity resulted in over 95% purity in secondary glial cultures.

The supernatant medium from the initial plating of the cell suspension was used to prepare homogeneous neuronal cultures. Following a second 15-min preparative plating to remove carry-over glial contaminants, a neuron-enriched fraction was obtained by plating the cells remaining in this second supernatant. Two 24-h treatments of 10^{-4} M cytosine-d-arabinoside (ARA-c, Sigma) proved to be effective in removing residual glial cells from these enriched neuronal cultures. The neuronal populations thus obtained were determined to have over 97% purity. The identification of neurons and glia was made on the basis of morphological criteria, tritated thymidine assays for mitotic activity, and histochemical assays for acetylcholinesterase and butyrylcholinesterase [49].

Pretreatment of culture dishes with poly-l-lysine (5 μg/ml; Sigma, mol wt 340,000) markedly facilitated the attachment of cells to the plastic surface [50] and was used routinely for both the sequential plating steps and the final purified preparations.

Early passage fibroblasts, prepared from 18-day mouse embryos according to previously published protocols [51] were grown in 100 mm Falcon tissue culture dishes containing 10 ml growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution). Upon reaching confluence, cells were subcultured using 0.25% trypsin and seeded at densities of 10^6 cells/100 mm plate.

Purification of Fibronectin From Fetal Bovine Serum

Fibronectin was purified from human plasma and fetal bovine serum (FBS) by affinity chromatography according to previously published methods [52,53]. Briefly, chromatography was carried out at room temperature using BrCN-activated Sepharose 4B (Pharmacia) coupled to gelatin (Sigma, type I). Purification was carried out by first passing 40 ml of plasma or serum through a Sepharose 2B column to remove minor contaminants having affinity for the Sepharose. The voided material was subsequently passed through the gelatin-sepharose column. The fibronectin was eluted off the column as a single peak with 4 M urea (Schwarz/Mann) in 0.05 M tris buffer, pH 7.5, and then dialyzed extensively against the same buffer at 4°C. Passing the unabsorbed fraction through a second gelatin-Sepharose column did not increase the yield of fibronectin. Eluting the column with 8 M urea also had no effect on total yield. Protein determinations conducted according to the method of Lowry [54], using bovine serum albumin standards, revealed FBS to contain approximately 62.5 μg/ml of fibronectin. This value, however, was observed to be variable, changing signi-

ficantly with different lots of FBS. Analyses of the various fractions in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis system, using 7.5% slab gels according to the method of Laemmli [55], revealed that the fibronectin band present in the starting material was not detectable in the void volume of the gelatin-Sepharose column, and that reduced samples of the affinity purified material migrated as a single band with an apparent molecular weight of approximately 220,000 daltons. Adjacent wells containing purified preparations isolated from human plasma and cell surface fibronectin extracted from intact cultures of chick embryo fibroblasts [43] revealed that the human and bovine plasma fibronectins comigrated while the cell surface fibronectin was displaced slightly toward the cathode.

Analyses of samples by gel double-diffusion in agarose using rabbit serum containing antibodies to human plasma fibrotectin gave strong reactions with both the human plasma and FBS starting materials and the affinity-purified preparations while yielding no detectable precipitin bands against the voided material of the gelatin-Sepharose column. These observations, taken together with the results obtained by electrophoretic analysis, established that the purification protocol did, in fact, remove all fibronectin from the starting material and that the purified material obtained was fibronectin.

The FBS from which the bovine fibronectin had been removed was subsequently reconstituted to its original protein concentration by Amicon filtration, dialyzed extensively against Tyrode's solution, Millipore filtered, and used in the preparation of cell growth medium.

Antisera

Rabbit serum that contained antibodies to the fibronectin of human plasma was prepared by injecting highly purified fibronectin. The serum fraction was immunoabsorbed with the supernatant of Cohn fraction I. The resulting high titer antiserum was monospecific, as determined both by immunoelectrophoresis and by direct binding of fibronectin antibodies to the appropriate band on polyacrylamide gels [56]. This antiserum was generously supplied by Dr. Lan Bo Chen. The interspecies cross-reactivity of antisera prepared against purified fibronectin has been reported elsewhere [57]. Twenty μl of this antiserum was absorbed with 100 μg of purified plasma fibronectin. Following centrifugation, the supernatant was tested for residual immunoreactivity in immunofluorescence assays.

Antisera against GFA, kindly supplied by Dr. Doris Dahl, was obtained by injections of purified GFA (isolated from human spinal cord) into rabbits. Immunoelectrophoresis of the resulting antiserum gave evidence of its monospecificity [10].

Immunofluorescence

For examination of cell surface-associated fibronectin, cell monolayers plated on poly-l-lysine-coated glass coverslips (12 mm, Kimble) were extensively washed in Hank's balanced salt solution (HBSS) with bicarbonate buffer, pH 7.4, and placed in a humidified chamber. Ten μl of fibronectin antiserum prepared from rabbits injected with human plasma fibronectin, diluted 1:80 with HBSS, was layered on the coverslips and incubated for 30 min at 37°C. After washing in HBSS, the coverslips were then layered with 10 μl of fluorescein isothiocyanate-conjugated (FITC) IgG antibody from goat anti-rabbit serum (Cappel), diluted 1:20 with HBSS. After a second 30 min incubation at 37°C, coverslips were again washed in HBSS and were subsequently fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Following fixation, the coverslips were washed in PBS and mounted on microscope slides with Elvanol. Parallel samples of unfixed cells were similarily prepared.

Control samples consisting of 1) cells incubated with normal rabbit serum in place of the specific antibody, 2) preparations in which the anti-fibronectin serum was omitted, and 3) coverslips exposed to normal goat serum prior to labeling with the FITC-conjugated antibody were also prepared. Fluorescence was observed using a Zeiss fluorescence microscope equipped with epi-illumination.

For intracellular visualization of GFA, coverslip preparations were fixed in formyl alcohol (9 parts 95% ethanol:1part 37% formalin) for 20 min at 4°C. Cold acetone (½ volume) was then added for 15 min, followed by a 30 min exposure to 100% acetone at 4°C. After extensive washing and treatment with Tween 80 detergent (Sigma, 3% aqueous solution) for 1 min at 20°C, the cells were again washed and subsequently labeled in the manner described above, using rabbit anti-human GFA serum, diluted 1:50 with PBS. Appropriate controls were carried out, as described above for the anti-fibronectin serum.

RESULTS

Morphology of Whole Cerebrum Cell Cultures

Following dissociation of cerebral tissue from chick or mouse embryos, the resulting cell suspension, when examined under phase-contrast microscopy, revealed single, phase-dark cells, many of which had prominent nucleoli. These cells could be grouped into two general size classes: 10–15 μ, and 3–7 μ in diameter. These observations were consistent with those reported by Varon and Raiborn [48], who used analogous cell systems. On the basis of evidence presented here and in previously published studies using identical or similar cell models [58–62], it appeared that the larger rounded cells were neuronal in origin, and that the smaller proliferative cells were glial precursors. By 72 hours, the small, nonaggregated cells (glioblasts) took on a flattened, spread-out appearance typical of epithelial morphology and exhibited irregular contours and many cytoplasmic granulations. These cells proliferated rapidly and achieved confluence by 7 days in culture.

During this period the neurons had increased in size and retained their rounded cell bodies, each with a clear nucleus and prominent nucleolus. The neuronal cells, mostly bipolar in appearance, aggregated into clusters. These aggregates invariably rested upon the underlying glial monolayer, suggesting a perferential affinity for the glial cells, or their exudates, as substrates rather than for the culture dish surface (Fig. 1A). Cell processes, first observed extending from neuronal aggregates after 24 h, fasciculated into fiber bundles, some of which were over 3 mm long after 7 days in culture. These nerve fibers formed an intricate meshwork which interconnected with other neuronal aggregates within the culture. Neuronal perikarya were found moving along the newly formed fascicles and the underlying glial monolayer.

Separation of Neuronal and Glial Cell Types

Separated homogeneous populations of glial cells and neurons were prepared from dissociated cerebral cell suspensions by exploiting a combination of differential adhesiveness to the culture dish during the initial plating and differential cytotoxic responses to the nucleoside analogue, cytosine-d-arabinoside, as described in Methods. Along with inhibition of DNA polymerase activity, ARA-c produced selective toxic effects on the actively dividing glial components, while apparently having no effect on neurons, which are postmitotic at this gestational age [58,63]. Examination of purified neuronal cultures after treatment with ARA-c revealed an absence of glial elements, while the neuronal aggregates and interconnecting fiber tracts remained intact (Fig. 1B). Poly-l-lysine pretreatment of neuron-

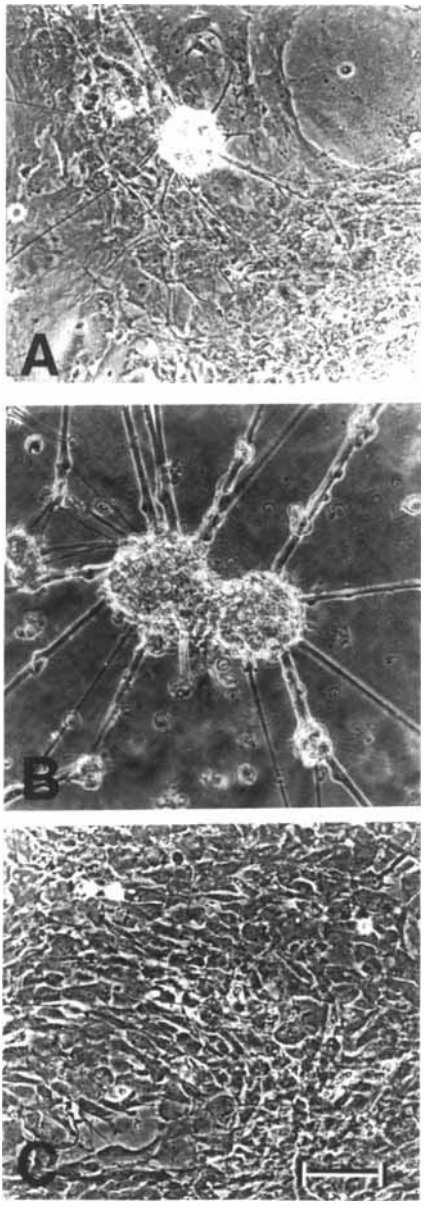

Fig. 1. Ten-day chick embryonic cerebral monolayer cultures, phase-contrast. (A) Mixed culture of neurons and glia after 7 days in culture. (B) Homogeneous neuronal culture after 7 days in culture. (C) Homogeneous glial culture after 7 days in culture (bar = 90μ: magnification, × 150).

al cultures was critical for attachment of neuronal elements to the surface of the culture dish, since the glial cells, which normally form the neuronal substratum, were absent.

Initial glial cultures obtained by rapid plating, although substantially enriched for glial cells, contained varying numbers of neuronal contaminants, which were effectively removed by subculture after the glia reached confluence. Phase microscopic examination of purified glial cultures obtained in this way revealed cells with an exclusively epithelial morphology (Fig. 1C).

Expression of GFA by Epithelial Components

Comparative immunofluorescent analysis of fractionated mouse monolayer cultures using rabbit serum containing antibodies to human GFA, revealed the presence of GFA in the epithelial glial cell fraction, and its absence in the neuronal fraction. Parallel preparations of glial cells treated with normal rabbit serum yielded no detectable specific fluorescence (Fig. 2C,D). Furthermore, mouse embryo fibroblasts similarly prepared were also GFA-negative (Fig. 2E,F). At early states of culture, only 5–10% of the glial cells expressed this protein; however, after 14 days in culture essentially 100% of the cells in the glial cultures were GFA-positive. The antigen was distributed within the cytoplasm as filamentous arrays, which were randomly oriented within the somatic portion of the cell. GFA protein within the filopodia consistently had a polarized configuration oriented parallel to the long axis of the process (Fig. 2A,B). These results confirmed the classification of these epithelial cells as glial by immunochemical criteria and demonstrated that differentiation of these cells progresses in vitro.

Distribution of Fibronectin

With antiserum directed against fibronectin, indirect immunofluorescent staining of embryonic chick and mouse cerebral cells in monolayer cultures containing both neurons and glia revealed that fibronectin, or a closely related molecule, was associated with the cell surface of many, but not all, of the cells. That fibronectin antiserum was reacting with an antigen of cellular origin, and not a serum contaminant, was established by growing the cells in culture medium containing serum from which the fibronectin had previously been removed (see Methods). Cells fixed in 2% paraformaldehyde following antibody labeling had immunofluorescent staining patterns identical to those of parallel live preparations. Protocols in which the cells were fixed before labeling,however, consistently introduced a low level of diffuse background fluorescence not evident in post-fixed or live preparations. Cells prepared in the manner described in Methods served to establish the localization of fibronectin at the external cell surface. Prior absorption of the fibronectin antiserum with purified human plasma fibronectin abolished all detectable specific fluorescence. Similarly, cells treated with pre-immune rabbit serum or FITC-conjugated goat anti-rabbit serum alone yielded no specific fluorescence.

Upon further examination, it was found that fibronectin expressed in heterogeneous cultures of cerebral cell populations could be detected as early as 24 h in culture. More significantly, fibronectin was localized to cells with a flattened epithelial morphology, indicative of glial origins. Moreover, the rounded neuronal elements did not express the antigen. Immunofluorescent examination of homogeneous glial cell cultures demonstrated that the glial cells were the source of fibronectin expression demonstrated earlier in the whole cerebral cell monolayers. In isolated glial cell cultures fibronectin increased in a strictly density-dependent manner. In sparse cultures there was little detectable fibronectin on the surface of the glial cells. However, it was noted that the fibronectin present in these cultures was

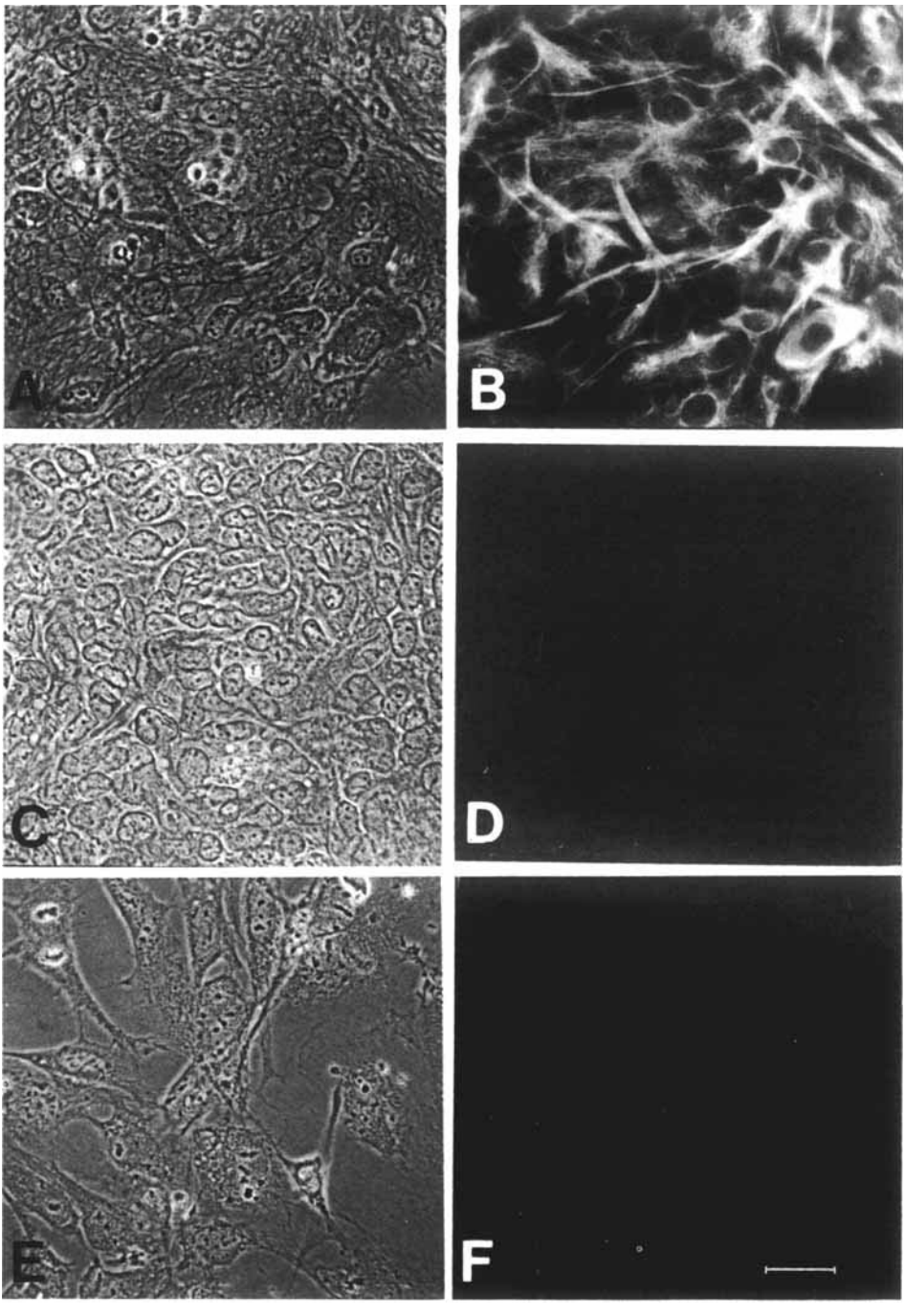

Fig. 2. Homogeneous glial monolayer after 14 days in culture, (A) viewed with phase-contrast; (B) corresponding field showing indirect immunofluorescent visualization of intracellular GFA; (C) parallel homogeneous glial preparation viewed under phase contrast; (D) corresponding field showing indirect immunofluorescent reaction with normal rabbit serum; (E) mouse embryo fibroblasts after 14 days in culture viewed under phase-contrast; (F) corresponding field labeled with GFA antiserum (bar = 10μ; magnification, × 400).

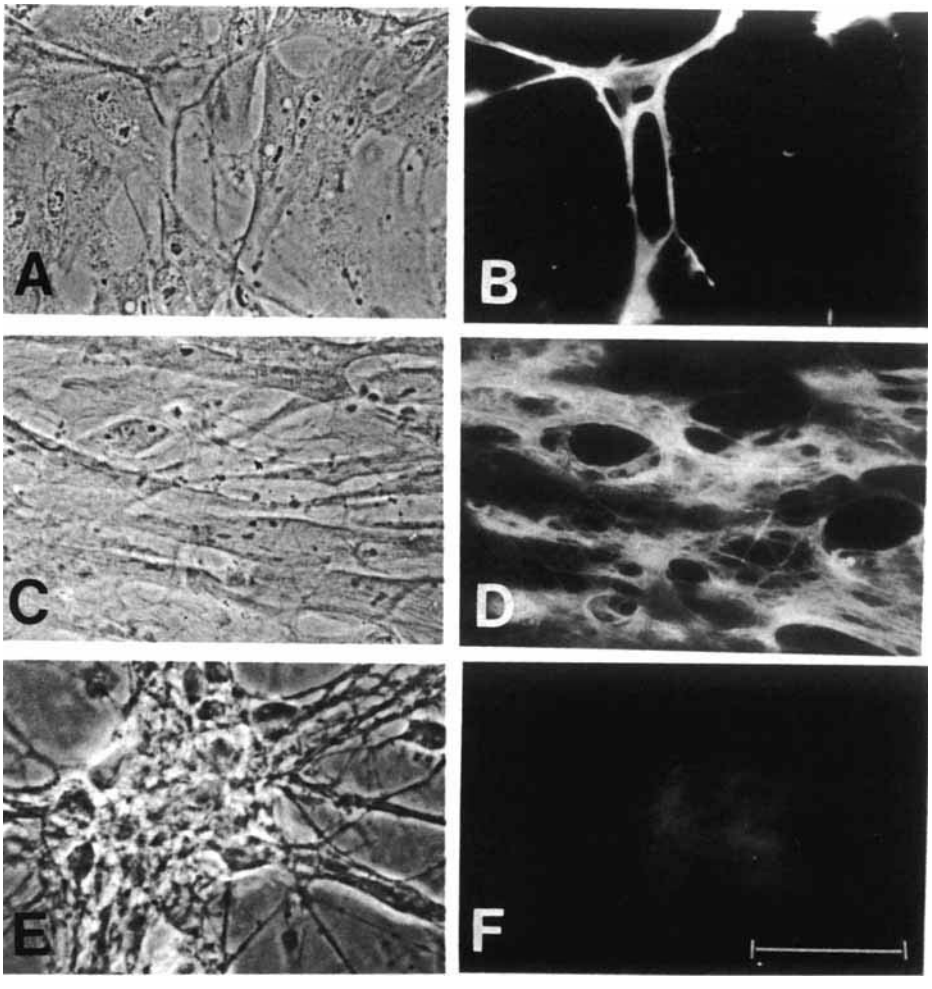

Fig. 3. (A) Purified glial cell fraction after 72 h in culture, phase-contrast; (B) corresponding field showing indirect immunofluorescent distribution of cell surface fibronectin; (C) homogeneous population of confluent glial cells after 7 days in culture, phase-contrast; (D) corresponding immunofluorescent staining of fibronectin. Note dense fibrillar-like network associated with cell processes; (E) purified neuronal monolayer culture after 7 days, phase-contrast; (F) corresponding field after staining with antifibronectin serum (bar = 30μ; magnification, × 400).

localized on filopodial-like processes (see Fig. 3A,B). With progressive proliferation, the cells became increasingly dense, and after 7–10 days reached confluence. Concomitantly, the fibronectin level increased and was observable as a dense matrix closely associated with the surface of the culture dish (Fig. 3C,D).

Glial cells grown in serum from which the fibronectin had been removed by affinity chromatography showed no alteration in their ability to attach and spread on the underlying substratum, nor in the pattern of fibronectin-specific fluorescence observed. While this result does not ensure that serum fibronectin will not bind to the cells in culture, it does indicate that most of the specific fluorescence observed is not attributable to serum-derived protein.

In contrast to the intense fluorescence seen in glial cell cultures with the anti-fibronectin serum, immunofluorescent examination of corresponding pure neuronal cultures demonstrated that the neurons were devoid of fibronectin (Fig. 3E,F). Thus, fibronectin is expressed by the glial component of developing cerebral cell populations and serves as a diagnostic marker of this cell type in vitro.

DISCUSSION

The results presented above suggest that fibronectin, a well-characterized cell surface glycoprotein, may provide a useful biochemical marker for developing glial cells grown in vitro. This is consistent with the recent demonstration of fibronectin in cultures of normal adult human glial cells [64]. While immunological data demonstrate that there are antigenic determinants shared by fibronectin and cell surface components on young glial cells, these tests do not permit direct identification of the cell surface antigen as fibronectin. However, the disposition, topographical arrangement, and general properties of the glial antigen – ie, density dependence and fibrillar appearance – are consistent with those expected for fibronectin.

Expression of fibronectin by glial cells, and its absence in neuronal elements, may represent a significant cell surface property governing neuronal–glial interactions during neurogenesis. In view of the ubiquitous nature of fibronectin, it is quite unlikely that it participates in the establishment of highly specific intercellular contacts. However, a general adhesive role for fibronectin cannot be ruled out. It is possible that this substance, expressed by immature glia during development, may serve to bring cellular elements into close opposition to one another in a nonspecific manner. Subsequently, adherent cells may further interact with one another in accordance with the histogenetic program. Similar mechanisms may be involved in facilitating the migrations of newly born neuronal cells to their final positions in the cephalic wall. This notion is not without precedent, since such a role for radial glia has been previously put forward by Rakic [65,66].

We have shown that neuronal cells in the absence of glia and of glial-associated fibronectin retain properties for mutual adhesion and aggregate to form tightly coherent cell clusters under these conditions. However, in the presence of glia, neurons tend to adhere to the glial substrate and to move away from their neuronal neighbors. Such neuronal–glial cell interactions, observed earlier in explant cultures [67,68] and monolayer cultures [60], suggest that fibronectin may play a role as an adhesive molecule, enabling neurons to extend pseudopodial outgrowths along a glial framework. In the case of freely moving neurons, this could result in the directed migration and translocation of neurons along predetermined pathways; in the case of anchored neurons, the elongation of axonal or dendritic processes could result in the alignment of cortical elements or the establishment of fibrous tracts.

The proposal that such a mechanism is operative in the intact embryo is, of course, contingent upon demonstrating fibronectin in brain tissues developing in situ. Recent observations by Schachner et al [69], using immunofluorescent assays, have indicated that fibronectin can be seen neither in intact adult nor embryonic tissues. However, these results may be due to the dynamic state of fibronectin in vivo – eg, a rapid turnover of fibronectin on glial cell surfaces in vivo or its appearance in a less polymerized form than that exhibited in

monolayer cell cultures. Further analysis of the dichotomous expression and behavior of this molecule in vivo and in vitro seems warranted, and investigations are in progress to address this question by more sensitive methods for detection of fibronectin in brain tissues both in situ and in 3-dimensional aggregates reconstituted in vitro.

Since the neoplastic counterparts of normal fibronectin-producing cells have generally been shown to be devoid of fibronectin [12,64], it is of interest to note that cerebral glial-derived cells of the C_6 astrocytoma line do not express fibronectin [70]. When dissociated C_6 astrocytoma cells were plated on a confluent sheet of embryonic glial cells, it was found that they failed to adhere to normal glia and, instead, penetrated between the normal glial cell junctions [71]. The astrocytoma cells then migrated underneath the normal glial sheet attaching preferentially to the culture dish surface. The possibility that neoplastic alterations of glial cell surface properties operative in tissue invasiveness may also involve fibronectin raises provocative questions. Not only may the absence of fibronectin on astrocytoma cells prevent cell–cell contacts and tissue relationships with normal glial cells, but, in addition, proteolytic activity by the neoplastic glial cells may also destroy the preexisting fibronectin mediated cell–cell and cell–substrate adhesiveness that preserves the integrity of the normal tissue.

In conclusion, the demonstration of fibronectin associated with the glial component of embryonic brain tissue may be related to the neuromesenchymal function of glia in neurogenesis, particularly in terms of the stromal support it provides for developing neuronal networks.

ACKNOWLEDGMENTS

We would like to thank Dr. Lan Bo Chen and Dr. Doris Dahl for providing anti-fibronectin and anti-GFA sera. Appreciation is also extended to Dr. Ken Yamada for his contribution of purified cell-extracted fibronectin. We are also grateful to Marion Sullivan for her secretarial assistance during the preparation of the manuscript.

This work was supported by NIH grants CA 19265, NS 10714, and GM 07151.

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