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BRIEF METHOD

A Novel Method for In Vitro Production of Human Glial-Like Cells from Neurosurgical Resection Tissue

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Progress in brain cell therapy requires the development and optimization of methods to provide a reliable supply of human neural cells. Isolation of astrocytes derived from adult human brain has led so far to poorly characterized cells because the identification of astrocytes is based solely on the expression of the astrocytic marker glial fibrillary protein (GFAP) (Barna et al, 1989; Ridet et al, 1999; Wu and Schwartz, 1998). In the method presented here, tissue preparation and culture conditions have been optimized and include an enrichment of cell suspensions from white matter (WM) and grey matter (GM) as well as a procedure for selection of appropriate fetal bovine sera (FBS) that consists of evaluating the ability to support neonatal mouse astrocyte primoculture. Long-term (up to 150 days) primocultures using adult human temporal lobe tissue from epilepsy or trauma neurosurgery patients were reliably obtained. Immunocytochemical characterization revealed that cultures mainly consisted of neuroectodermal cells presenting some functional characteristics of astrocytes, such as glutamate uptake.

A method to produce substantial numbers of glial-like cells using small amounts of adult human brain tissue (approximatively 100 mg) as starting material was developed by adapting a procedure used to prepare neonatal mouse cortical astrocytes (Pellerin and Magistretti, 1994). We have found that a useful and predictive test to select a suitable serum for human cultures is to determine its ability to support neonatal mouse cortical astrocyte cultures. Briefly, neonatal mouse cortices were dissected, mechanically dissociated, and cells seeded at a density of 100,000 cells/cm^2 in DMEM with 10% FBS. After 20 days in vitro, cells were washed and resuspended in lysis buffer NaOH 10 mM + Triton X-100 0.1%, and protein concentration was measured with BCA protein assay reagent (23225; Pierce Chemical, Rockford, Illinois). Of five sera tested, three that allowed mouse astrocyte cultures to grow near confluency after 20 days in vitro with a protein concentration range of 150 to 200 μg per dish (diameter 3.5 cm) also supported the growth of cells derived from human brain tissues (Table 1). In contrast, two other sera that gave poor results with mouse cultures (either no proliferation or few cells with a protein concentration range of 50 to 100 μg per dish) were unable to support cultures from human tissues. Thus, it is suggested that FBSs are suitable for the preparation of adult human brain cell cultures, but their efficacy has to be evaluated first on neonatal mouse cortical astrocyte cultures.

Pieces of cerebral cortex were obtained from epilepsy (n = 21) or trauma (n = 3) neurosurgery patients in accordance with ethical committee guidelines. All tissues came from the temporal lobe (right, 20 and left, 4). Tissues were dissected with a razor blade to obtain enriched fractions of WM and GM. For two independent material sources, results show that only enriched WM or GM cell suspensions led to substantial numbers of cultured cells, as opposed to whole temporal cortex that did not.

For each fraction, primary cultures were generated by mincing and mechanically triturating the tissue with fire-polished glass pipettes of decreasing diameters. Cells were plated at 250,000 cells/cm^2 in D7777 medium (Sigma, Buchs, Switzerland) supplemented with $NaHCO_3$ 44 mM, 10% or 20% FBS, and an antibiotic/antimycotic cocktail (A7292 Sigma) directly on glass coverslips for immunocytochemistry or on plastic dishes and incubated at 37° C in a water-saturated atmosphere containing 6.5% CO_2/93.5% air. It appears that molecules such as polyornithine or polylysine impair rather than improve survival and/or proliferation of adult human brain cells under the conditions selected. Fifteen days later, the concentration of serum was reduced to 10%. When cells became confluent between 30 to 45 days, medium was

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Table 1. Selection of Serum for Human Adult Brain Cell Culture

		Horse serum 380S		Human serum		Fetal Bovine Serum (FBS)			
		10%	5% + FBS*	5% + FBS*	2.5% + FBS*	264S 10%	524X 10%	541U 10%	654X 10%
Neonatal mouse astrocyte*		ND				Cell 150–200 μg	Cell 150–200 μg	Cell 50–100 μg	No cell
Human brain cell	4a 5a	No cell				Cell 50–100 μg	ND		
	32a 33a 37a	ND					Cell 100–150 μg	No cell	No cell

ND, not determined.

* 10% FBS (lot 264S).

Note that human brain cells have been successfully obtained with another serum (Brunschwig 99145), which seemed to give results comparable to sera biochrom 264S and 524X.

replaced with one without serum and cells were maintained in these conditions for a maximum of 157 days. Four cell cultures were prepared in parallel with either mechanical or enzymatic dissociation, using papain in two cases and trypsin in two other cases. Cells from enzymatic dissociation required a longer time to be confluent, 60 to 80 days versus 30 to 45 days for the mechanically dissociated cultures. The enzymatic treatment seems to modify cell growth, but does not alter the morphology of cells or their general phenotype.

Overall, using optimized conditions (mechanical dissociation, preparation of enriched fractions of WM or GM seeding on uncoated culture dishes, use of selected FBS), we succeeded in preparing long-term primocultures of human brain cells from adult temporal lobe tissues obtained from epilepsy ($n = 21$) and trauma ($n = 3$) neurosurgery patients with a success rate of 100%.

Observation of cultures with phase contrast microscopy or with light microscopy after fixation and staining with hematoxylin revealed particular morphologic characteristics. From the beginning, cultured human adult brain cells appeared as a heterogeneous population and first presented two distinct morphologic patterns: flat cells with a large nucleus and important cytoplasm or stellate cells with a small nucleus and long processes (Fig. 1, a and b). After cells reached confluency, bundles of closely associated cells formed and evolved into a dense network (Fig. 1, i to k) on top of flat cells.

Characterization by immunocytofluorescence revealed that virtually all cells stained for GFAP (monoclonal G3893 [Sigma] and polyclonal Z0334 [DAKO, Glostrup, Denmark]), with stellate cells being more intensely labeled than flat cells (Fig. 1, c and l). Interestingly, we observed that isolated stellate cells that expressed GFAP also coexpressed beta-tubulin III (T8660 Sigma) (Fig. 1, c to e) and the oligodendrocyte marker O4 (Roche Diagnostics, Rotkreuz, Switzerland) (Fig. 1, f to h). Before confluency (up to 30 to 40 days in vitro [DIV]), neither vimentin (3B4 M7020; DAKO)- nor nestin- (a generous gift from Ron DG McKay; Tohyama et al, 1992) positive cells were observed (data not shown). As the culture evolved and bundles formed, three distinct phenotypes could be distinguished: flat cells that slightly expressed GFAP, small closely packed cells that constitute the core of the bundles and express vimentin (Fig. 1m) and/or nestin (data not shown), and finally stellate cells that present long GFAP-positive processes oriented longitudinally and that are associated with bundles (Fig. 1, l to n). The emergence of vimentin- and nestin-positive cells within cell bundles suggest that an ongoing undifferentiation process is taking place in these cultures. However we cannot completely exclude the possibility that a few early, undetected nestin-positive cells gave rise to this later expansion. Based on the pattern of expression of these four markers (GFAP, beta-tubulin III, O4, and vimentin), no significant phenotypic or morphologic differences could be observed between cells prepared from GM and WM at any time point. In relation to the presence of GFAP-positive cells, we functionally tested their ability to transport glutamate by measuring the uptake of ^{3}H-D-aspartate as previously described for neonatal mouse astrocyte (Debernardi et al, 1999). Briefly, cells were incubated for 5 minutes with fresh medium containing ^{3}H-D-aspartate at a tracer concentration of 1 μCi/ml (47 to 60 nM) and unlabeled D-aspartate at the indicated concentration. Cells were then washed three times with ice-cold phosphate buffer and lysed with 2 ml per well of 10 mM NaOH + 0.1% Triton X-100. Aliquots of 250 μl were assayed for radioactivity by liquid scintillation counting, and 30 μl aliquots were used for protein concentration measurement. A small, active transport of glutamate was reliably detected, which represents another feature associated with mature glial cells (Danbolt, 2001; Fig. 2).

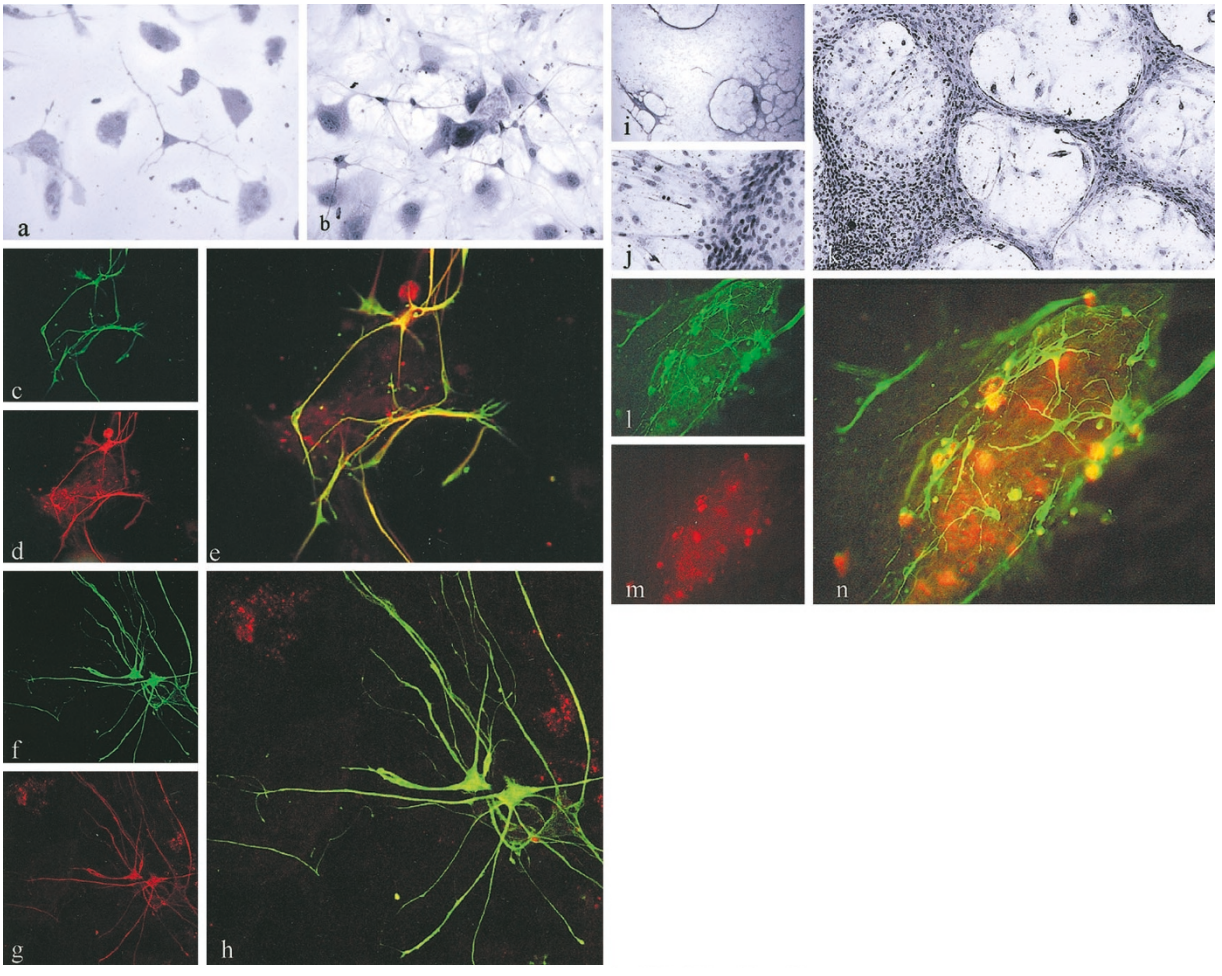

Figure 1.

Cytology of adult human brain cells. a, b, and i to k, hematoxylin staining of the same mechanically dissociated culture 5a at different times: a, 15 days in vitro (DIV); b, 38 DIV; i to k, 67 DIV. c to e, glial fibrillary protein (GFAP) (green) and beta tubulin III (red) colocalization in stellate cells in the mechanically dissociated culture 5a at 38 DIV. f to h, GFAP (green) and O4 (red) colocalization in stellate cells in the enzymatically dissociated culture 7a at 71 DIV. l to n, expression of GFAP in long processes of stellate cells (green) and coexpression of GFAP (green) and vimentin (red) in closely aggregated small cells in the mechanically dissociated culture 5a at 67 DIV. Original magnification: a to h, j, and l to n, ×40; i, ×10; k, ×20.

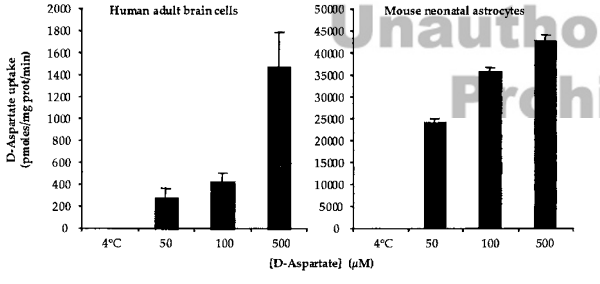

Figure 2.

^{3}H-D-Aspartate uptake on human adult white matter cell culture that had already presented bundles, and for comparison on neonatal mouse astrocytes at 21 DIV.

This novel method offers a potentially prolific source of human glial-like cells that could be useful to characterize the function of astrocytes in neuropathologic conditions, as well as in the context of new neurosurgery strategies such as autologous cell therapy, in combination with ex vivo gene transfer (Ridet et al, 1999). In addition to potential clinical applications, such preparations might also provide a valuable tool for studying human brain cell biology, an opportunity that might benefit the development of future therapies. Among the next challenges to address, it will be important to determine how to control the fate of these cells, to characterize their engrafting abilities, and to follow their development in vivo to improve and adapt the autografting strategy for each neurologic disease concerned by this approach in the future.

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