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Analysis of the temporal expression of nestin in human fetal brain derived neuronal and glial progenitor cells

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Abstract

Nestin expression in the developing human brain was examined with the use of unique human specific anti-nestin antibodies. Double immunostaining of cell cultures and tissue sections derived from first and second trimester human fetal brain (HFB) examined the co-expression of nestin with other cell type specific phenotypic markers. The immunocytochemical analysis shows that from first to second trimester, the majority of developing glial cells exhibited a transitional state marked by co-expression of nestin and GFAP. However, the corresponding transitional state for developing neuronal cells, co-expressing nestin and MAP-2, was rarely detected. These results imply different temporal patterns of nestin expression in cells of glial and neuronal lineages. Confocal microscopy of HFB tissue section staining also revealed a similar pattern of nestin co-expression with glial and neuronal markers. Our results suggest that nestin expression alone may not identify an undifferentiated stem cell, and that progenitor cells in glial and neuronal lineages express nestin in different temporal patterns. Published by Elsevier Science B.V.

Theme: Development and regeneration

Topic: Glia and other non-neuronal cells

Keywords: Stem cell; Glia; Astrocyte; Astrocyte progenitor; Neuronal progenitor; GFAP

1. Introduction

Nestin is a 220 kDa class VI intermediate filament protein originally found to be expressed early in central nervous system (CNS) development [12]. Initially, nestin expression appears during the time of CNS stem and progenitor cell proliferation and neuronal migration, and subsequently decreases as the brain develops [6,10,16,29,30]. Although nestin is expressed in undifferentiated CNS precursors, little information is available about the temporal expression of nestin as these cells differentiate into either a neuronal or glial lineage. It has also been suggested that nestin may not always be associated with stem/progenitor cells in the CNS [6,28]. Nestin has been detected in neuroectodermal and glial cell-derived tumors [5,29] as well as in reactive gliosis [15,11,3,18,17]. The production and characterization of human-specific rabbit polyclonal [20] and mouse monoclonal nestin antibodies [21], recently developed by this laboratory, has allowed for the detection of nestin expressing cells in the developing human CNS.

In this study, we sought to investigate the expression pattern of nestin in first and second trimester human fetal brain (HFB) cultures and brain tissue sections. The human-specific nestin antibodies, used in conjunction with existing cell-type specific phenotypic markers, were utilized to determine the phenotypic profile of nestin expressing cells in the normal developing human CNS. This is the first study, to our knowledge, to specifically examine nestin expression in cells of neuronal and glial lineage using both cultured cells and brain tissue sections. The observation that nestin is co-expressed with glial or neuronal markers suggests that nestin expression alone may mis-identify

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cells as undifferentiated progenitors when, in fact, such cells may have already begun a differentiated pathway.

2. Methods

2.1. Cell culture

Human fetal brains were obtained and processed as previously described [9] in accordance with guidelines at the National Institutes of Health. Cells were grown in EMEM supplemented with 10% fetal calf serum, glutamine (2 mM), amphotericin B (0.5 μg/ml), gentamycin (50 μg/ml) and penicillin/streptomycin (100 i.u./ml) at 37 °C with 5% CO_2. Dissociated cells from each HFB sample were cultured for 5 days before immunocytochemistry was performed. Cell culture products were obtained from Mediatech (Herndon, VA, USA) and Quality Biologicals (Gaithersburg, MD, USA).

2.2. Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde and permeabilized with 0.02% Triton. The fixed cells were then incubated with mouse monoclonal antibodies against human nestin (1:100 dilution of hNestin 10C2) generated by this laboratory [21], vimentin (1:100 dilution) CD68 (1:25 dilution) from Dako (Carpinteria, CA, USA), GFAP (1:25 dilution), O4 (1:25 dilution), A2B5 (1:100 dilution) from Boehringer Mannheim (Indianapolis, IN, USA), microtubule associated protein-2 (MAP-2, 1:200 dilution) from Calbiochem (San Diego, CA, USA), or β-III tubulin (1:500 dilution) from Covance (Richmond, CA, USA), at room temperature for 60 min. Fluorescein conjugated goat anti-mouse secondary antibody (1:100 dilution; Jackson ImmunoResearch, West Grove, PA, USA) was then added for 60 min. A second set of rabbit polyclonal primary antibodies against human nestin (1:200 dilution of Nestin 331B) [20], GFAP (1:100 dilution), or fibronectin (1:100 dilution) from Dako was then added for 60 min followed by rhodamine conjugated donkey anti-rabbit secondary antibody (1:100 dilution, Jackson ImmunoResearch) for 60 min. Control immunostaining was performed without primary antibodies. Cells were then incubated for 10 min with bisbenzimide (5 ng/ml) to label the nuclei of all cells. For cell counting and photographs, a CCD camera connected to the Ziess axiovert S100 microscope, with appropriate fluorescence filters, was used to capture four random fields of approximately 100 cells for each antibody staining combination, done in duplicate. The immunopositive cells from five 8- or 9-week gestation HFB (first trimester) and four 16-week gestation HFB (second trimester) were counted. These HFB cell cultures essentially are not reactive, since they produce very low levels of TNFα and prostaglandins, and can be activated by PMA, IL-1β and HIV tat protein [4,7]. The majority of human fetal brain collected for this study was from the telencephalon. Although it was not possible to obtain brain tissue from identical anatomical areas at each collection, great care was taken so that similar HFB regions were used for immunostaining of cultures and tissue sections.

2.3. Immunohistochemistry and confocal microscopy

HFB specimens were fixed overnight in neutral buffered formalin (37%), pH 7.4 at 4 °C, and equilibrated in graded sucrose. The tissues were embedded in low melting temperature paraffin, Paraplast X-tra (Fischer Scientific Pittsburgh, PA, USA), and 8 μm thin sections were cut and mounted onto poly-L-lysine coated glass slides. Prior to immunohistochemistry, sections were deparaffinaized in xylene, washed, and exposed to primary antibodies against human nestin, GFAP, and MAP-2 with appropriate fluorescent secondary antibodies, as described above. Hematoxylin and eosin staining was used to evaluate tissue sections before immunostaining. Several sections from seven 8–9-week gestation (first trimester) HFB and five 15–16-week gestation (second trimester) HFB were examined by immunostaining. Histological images were acquired with a Zeiss Axiovert LSM 410 laser-scanning confocal microscope station equipped with a krypton–argon laser (Thornwood, NY, USA) and appropriate fluorescence filters.

3. Results

Immunocytochemistry was performed on cultured HFB using various phenotypic markers to identify progenitor cells, astrocytes, neurons, oligodendrocytes, microglia and fibroblasts. Double and triple immunofluorescence staining was performed using either the mouse monoclonal or rabbit polyclonal anti-nestin antibodies in combination with other antibodies against cell-type specific phenotypic markers, in order to combine antibodies raised in different host species. The percentage of the total cell population immunostaining for each phenotypic marker is shown in Table 1. In first trimester HFB cell cultures, nestin positive cells comprised 56% of the cell population, which decreased to 32% in second trimester HFB. GFAP-positive astrocytes, however, showed an increase from 17% in the first trimester, to 36% in the second trimester. A similar increase in the percentage of MAP-2 positive neurons was also observed (10 to 43%) from first to second trimester. The results of A2B5 staining largely mirrored those of MAP-2. Based on morphology and co-staining with neurofilament antibodies, most A2B5 immunopositive cells in this study were determined to be neuronal (data not shown). Vimentin was detected in several cell types, co-expressing in cells also staining for nestin, GFAP, CD68, and fibronectin. A small percentage of cells expressing oligodendrocyte marker (O4) and microglial markers

Table 1
Percentage of total population of cultured HFB cells immunopositive for selected cell type specific phenotypic markers

Marker	First trimester (%)	Second trimester (%)
Nestin	55.7±7	31.9±9
MAP-2	9.8±7	43.4±21
A2B5	15.0±9	45.6±24
GFAP	17.4±11	35.6±15
Vimentin	45.6±13	48.5±14
O4	0.1±0.2	0.4±0.8
CD68	0.4±0.3	2.0±0.6
Fibronectin	3.0±4	2.0±1

Cells from five first trimester HFB and four second trimester HFB were immunostained and counted after 5 days in culture. Between 3000 and 10 000 cells were examined for each antibody used to stain each brain sample. Data represent mean (±S.D.).

(CD68) were found in both first and second trimester brain cultures.

Table 2 shows the percentages of the total HFB cell population co-expressing nestin and neuronal or glial phenotypic markers. A greater percentage of the astrocyte progenitors, defined by co-expression of nestin and GFAP, was detected in cultured second trimester HFB than in the first (Table 2). Conversely, the small percentage of neuronal progenitors, defined by co-staining for nestin and MAP-2, was higher in first trimester cultures than in the second (Table 2). Further analysis was conducted focusing only on the phenotypic profile of the nestin positive cell population. In first trimester HFB cultures, 11% of the nestin positive cell population co-expressed GFAP and 4% co-expressed MAP-2; the remaining majority of the nestin positive population (85%) expressed nestin alone. Conversely, in second trimester HFB cultures, 72% of the nestin positive cell population co-expressed GFAP and 1% co-expressed MAP-2, leaving only 27% of the nestin positive population expressing nestin alone. Both nestin antibodies and GFAP antibodies used in these experiments yielded the same pattern of nestin expression and co-expression of nestin and GFAP, respectively. The small percentage of nestin positive cells co-expressing neuronal markers was also confirmed using antibodies against β-III tubulin (data not shown).

To determine if the pattern of nestin co-expression with glial and neuronal markers in vitro reflected those in vivo, immunohistochemistry and confocal microscopy was performed on several first and second trimester HFB tissue sections. Double and triple immunostaining was performed using antibodies against nestin along with antibodies against GFAP, MAP-2 or β-III tubulin. Results showed that the cultures and tissue sections showed similar temporal patterns of nestin expression in developing glial and neuronal progenitor cells. As in the in vitro cultures, the majority of nestin positive cells from first trimester HFB tissue sections did not co-express GFAP (Fig. 1), while second trimester HFB tissue sections demonstrated a majority of nestin positive cells costaining with GFAP (Fig. 2). Most nestin positive cells from both first and second trimester HFB tissue sections were radial in morphology and located throughout the parenchyma of the brain. Nestin stained cells were also concentrated in the subventricular zone regions. In first and second trimester HFB tissue sections, cells co-staining with nestin and GFAP were largely radial in morphology. However, in second trimester HFB tissue sections there was the emergence of cells located in the brain parenchyma with differentiated astrocyte morphology, co-expressing nestin and GFAP. Nestin and MAP-2 co-positive neuronal progenitor cells were rarely detected in either first or second trimester HFB tissue sections (Fig. 3). The β-III tubulin-staining pattern on adjacent HFB sections was virtually identical to that of MAP-2 (data not shown).

4. Discussion

Previous studies have examined phenotypic markers [1,14,26,27] and described nestin expression [2,28,29] in the developing human CNS. However, this study specifically examined the phenotypic profile of nestin expression in human glial and neuronal lineage progenitor cells, both in culture and in tissue sections. The relative percentages of neurons, astrocytes, oligodendrocytes, and microglial cells determined in this study are consistent with previously published work [23,24,26,27]. HFB cultures demonstrated a twofold percentage increase in GFAP-positive astrocytes from first to second trimester (Table 1), accompanied by a corresponding increase in percentage of cells co-positive for nestin and GFAP (Table 2). This suggests that astrocytic lineage cells go through a transitional period during which they express both progenitor and astrocytic markers, with progenitors losing nestin expression as the brain develops, as previously described [2,20,28].

In these HFB cell cultures, a different pattern of nestin co-expression with GFAP or MAP-2 was observed between first and second trimester. Of the nestin positive

Table 2
Percentage of the total population of cultured HFB cells coexpressing phenotypic markers that indicate neuronal or glial progenitor cells

Cell types	Markers	First trimester (%)	Second trimester (%)
Neuronal progenitors	Nestin+/MAP-2+	2.3±3	0.8±1
Astrocyte progenitors	Nestin+/GFAP+	6.3±10	22.0±10

Cells examined in Table 1 were double or triple immunostained, with the percentages of co-positive cells shown. Data represent mean (±S.D.).

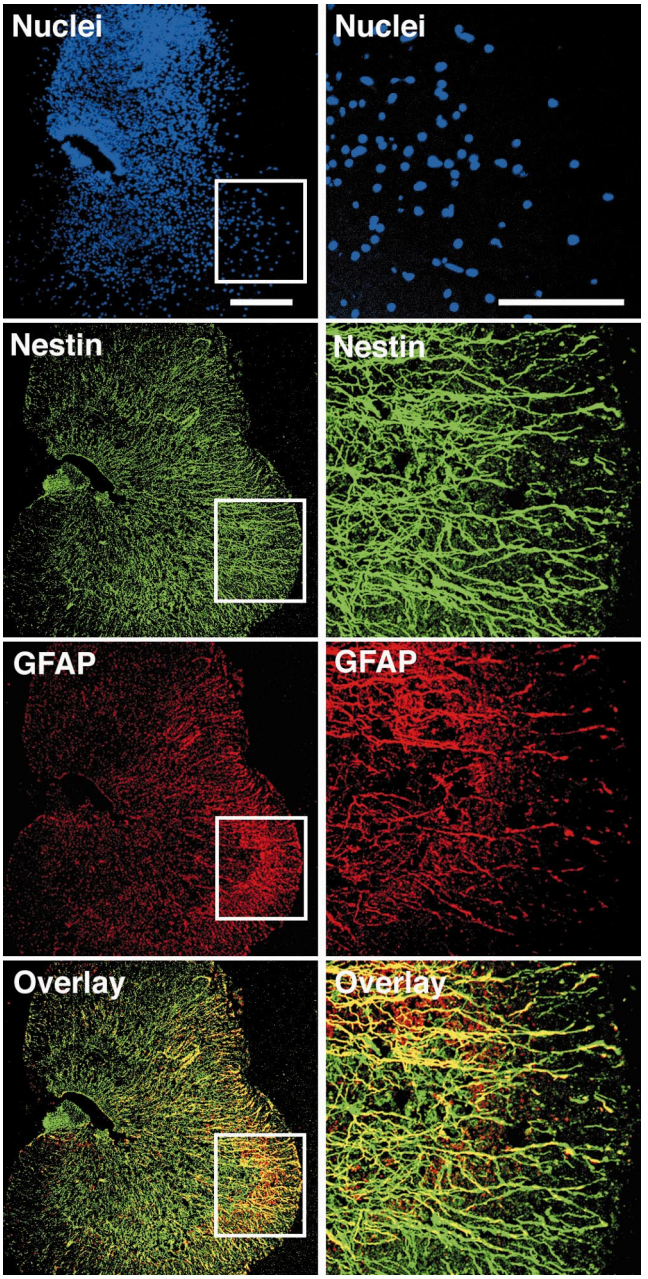

Fig. 1. Confocal micrographs of a 8 μm thick, 9-week gestation first trimester HFB tissue sections stained for nestin (green), GFAP (red), and nuclei (blue). Each column of images shows the same tissue section. The column to the right represents a higher magnification view of the area shown by the white box. The bottom panels are overlays of nestin and GFAP staining. Scale bar in upper left panels is 250 μm and in the upper right panels is 125 μm.

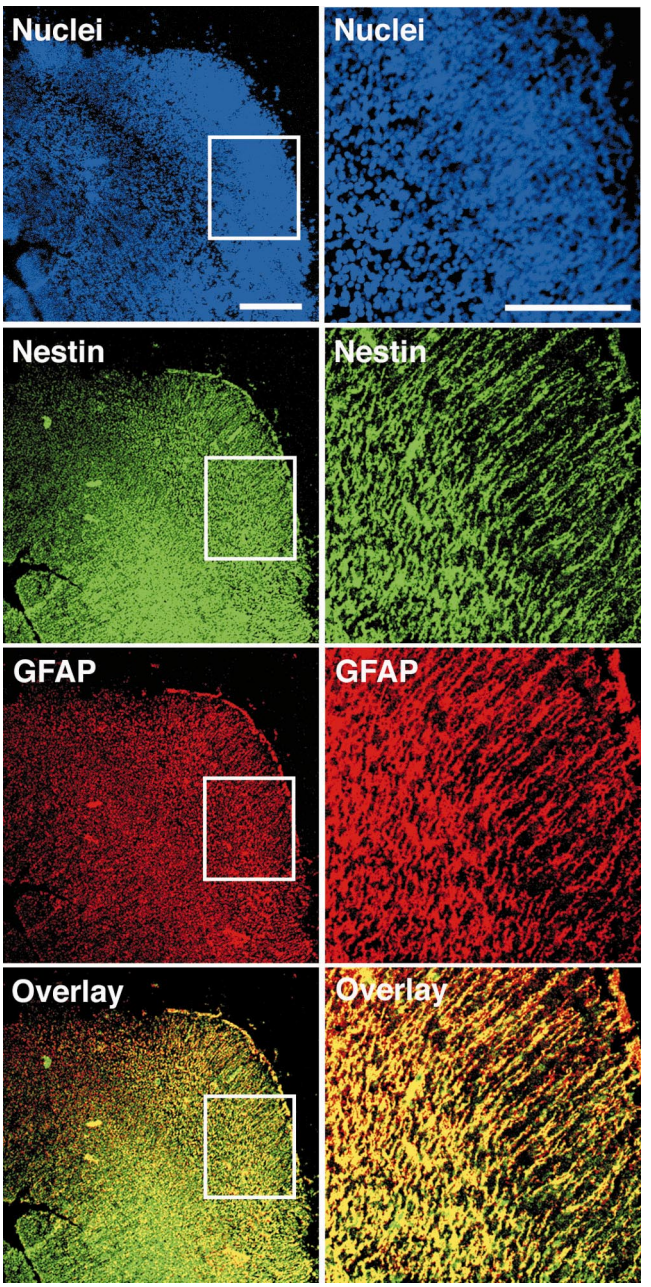

Fig. 2. Confocal micrographs of a 8 μm thick, 16-week gestation second trimester HFB tissue sections stained for nestin (green), GFAP (red), and nuclei (blue). Each column of images shows the same tissue section. The column to the right represents a higher magnification view of the area shown by the white box. The bottom panels are overlays of nestin and GFAP staining. Scale bar in upper left panels is 250 μm and in the upper right panels is 125 μm.

population found in first trimester cultures, 11% co-expressed GFAP and 4% co-expressed MAP-2. The majority (85%) of the nestin positive cell population in first trimester HFB was positive for nestin alone, suggesting that these cells are still undifferentiated and can presumably differentiate into astrocytes and neurons. Of the nestin positive cells found in second trimester HFB, only 27% were positive for nestin alone, while the majority of the population co-expressed GFAP (72%). These results further suggest a transitional state for a population of HFB cells marked by co-expression of nestin and GFAP.

Similar to astrocytes, the neuronal cell population demonstrated a fourfold percentage increase from first to second trimester. However, there was no corresponding increase in the percentage of cells co-positive for nestin

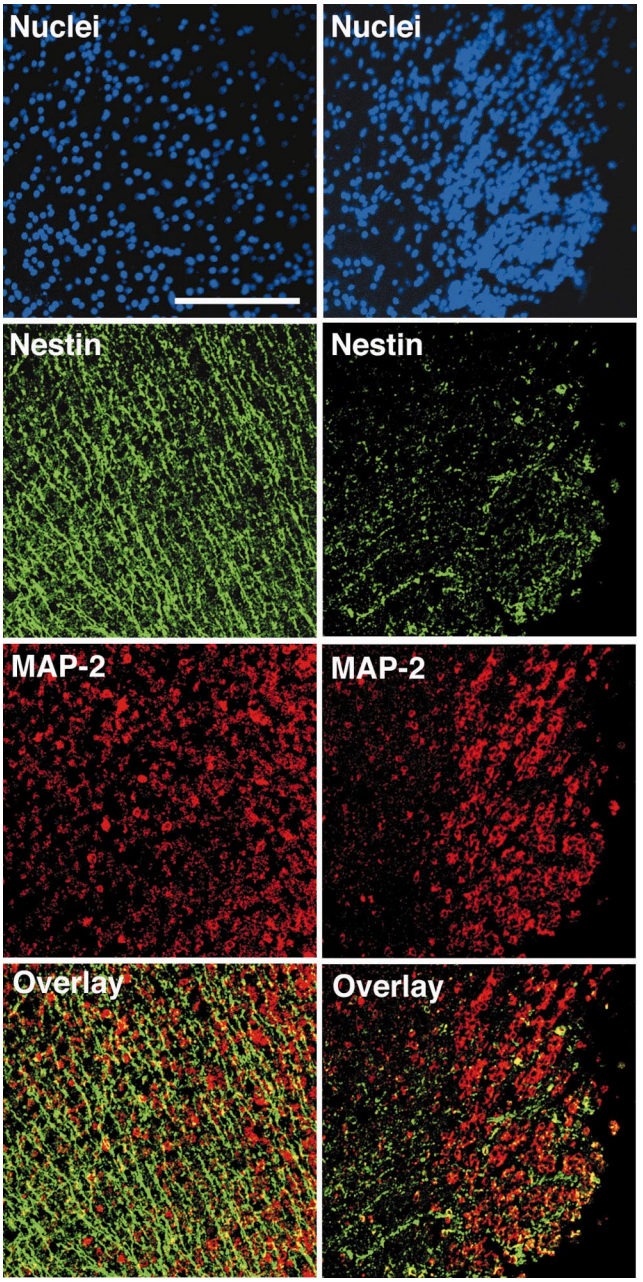

Fig. 3. Confocal micrographs of 8 μm thick, first and second trimester HFB tissue sections stained for nestin (green), MAP-2 (red), and nuclei (blue). Each column of images shows the same tissue section. The column on the left represents the immunostaining of a 9-week gestation first trimester HFB, and the column on the right is the immunostaining of a 16-week gestation second trimester HFB. The bottom panels are overlays of nestin and MAP-2 staining. Scale bars in upper left panels is 125 μm.

and MAP-2 in cultures (Table 2) nor tissue sections of HFB (Fig. 3). Unlike the astrocyte population, where co-expression of progenitor and glial markers could be easily detected, the transitional neuronal progenitor cells, positive for both progenitor and neuronal markers, were rarely detected. Staining with an alternative immature neuronal marker, β-III tubulin, also showed a very small percentage of neuronal progenitors co-expressing nestin in tissue sections and cultures of HFB (data not shown). These results suggest that neuronal lineage cells express nestin in a different temporal pattern than those in the astrocyte lineage. Based on our data, the possibility arises that nestin expression is down regulated before the expression of neuronal phenotypic markers such as MAP-2 or β-III tubulin. It is also possible that the neuronal progenitors express a different form of nestin not detectable by the antibodies used in this study. Alternatively, neurogenesis may be a result of the differentiation of a subpopulation of glial progenitors or radial glial cells, as previously hypothesized [8,13,22].

HFB cells in this study were cultured in serum-containing media; therefore it is possible that these conditions differentiate progenitors to astrocytes. However, the findings for cultured HFB are supported by a similar pattern of nestin co-expression with GFAP and MAP-2 or β-III tubulin observed in tissue section staining. It was not possible to quantitate the number of cells expressing nestin or GFAP in these tissue sections because of the long radial morphologies and extensive processes expressing these intermediate filaments. Still the co-expression pattern could be readily observed by confocal microscopy. Additionally, immunostaining with both the mouse monoclonal and rabbit polyclonal anti-nestin antibodies yielded the same results in cultures and tissue sections of HFB.

Although it is well established that nestin is expressed in undifferentiated CNS progenitor cells, this study is the first to suggest that nestin is expressed differentially in cells developing in glial and neuronal pathways. The implication of a varied temporal expression of nestin raised the possibility that there are molecular and or biochemical mechanisms that regulate nestin differently in these two cell populations. Indeed, there is evidence that enhancer elements in the nestin second intron can direct reporter gene expression differentially in CNS and muscle tissue [19,31]. Similar regulatory mechanisms may exist for neuronal and glial lineage cells. More recently, it was demonstrated that nestin is phosphorylated by cdc2 kinase during the cell cycle, a potential biochemical regulator of nestin expression [25]. An important observation of this study is that the presence of nestin alone does not determine an undifferentiated stem or progenitor cell. Some nestin expressing cells may have already begun to differentiate along a neuronal or glial lineage. Understanding the mechanisms that regulate nestin expression could provide essential information about cells committing to a neuronal or glial pathway.

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