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Biomarkers of glial cell proliferation and differentiation in culture

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1. ABSTRACT

The two major intermediate filament (IF) proteins of astrocytes are vimentin and GFAP. Early during development, radial glia and immature astrocytes express mainly vimentin. Towards the end of gestation, a switch occurs whereby vimentin is progressively replaced by GFAP in differentiated astroglial cells. The expression of vimentin and GFAP increased markedly after injury to CNS. GFAP has been widely recognized as an astrocyte differentiation marker, constituting the major IF protein of mature astrocyte. In our recent researches we investigated the interactions between growth factors and dexamethasone on cytoskeletal proteins GFAP and vimentin expression under different experimental conditions. In addition, nestin, a currently used marker of neural stem cells, is transiently co-expressed with GFAP during development and is induced in reactive astrocytes following brain injury. The role of S100B in astrocytes, neurons, and microglia is particularly studied in Alzhèimer's disease. In conclusion, such glial biomarkers will help us to understand the more general mechanisms involved in CNS development and can open new perspectives for the control of the neurologic diseases.

2. INTRODUCTION

The vertebrate nervous system is formed mainly by two heterogeneous classes of cells which are neurons and glia. Since the description and acceptance of the Neuron Doctrine nearly a century ago, neurons were regarded as the unique functional element of the nervous system. Glial cells, of which astrocytes constitute an important part, were described by Virchow in the middle of the nineteenth century as a merely supportive structural element of the nervous system. The astrocytes comprise a heterogeneous family of morphologically and functionally distinct cells whose structural plasticity is maintained mostly by a filamentous network consisting mainly of vimentin and GFAP. Since the description of astrocytes, the idea of a passive structural element of Virchow has been gradually replaced by a more dynamic picture in which the glial network may actively take part in neuronal physiology. Evidence accumulated in the past years demonstrates that astrocytes have a variety of active roles in maintaining normal brain physiology such as secretion of several active compounds, formation of the blood brain barrier, metabolism of several neurotransmitters and maintenance of the ionic balance of the extra cellular

medium (1).Radial glia, an astrocyte precursor, is involved in neuronal migration during embryonic brain development (2). Glial cells, in general, are also involved in providing neurotrophic signals to neurons required for their survival, proliferation and differentiation (3, 4).

Besides their physiological role, astrocytes play an important role in pathological conditions of the nervous system. Accumulation of glial fibers is the histological landmark of the astrocyte response to NS injury, appropriately named reactive gliosis. Such response is characterized by intense astrocyte proliferation and over expression of GFAP, among others. The fact that glial scars are frequently found in several NS disorders, such as multiple sclerosis, heavily implicates the astrocytic cells not only in the physiology of the NS as well as in neural disease development (5).

The cytoplasm of animal cells is structured by a scaffolding composed of microfilaments (MF), microtubules (MT) and intermediate filaments (IF). MF and MT are constituted by actin and tubulin, respectively, in all types of cells. Unlike MT and MF, IF proteins are regulated developmentally and tissue-specifically. The functional basis of this diversity is not well understood; however, changes in IF expression occur sequentially, coincidentally with changes in cellular differentiation states.

The differential expression of IF proteins in different cell types implies that they have specialized roles in the differentiation and function of these cells. At present, proteins that make up the IF family can be classified into five distinct types. Class I and class II IFs consist of acidic and neutral-basic cytokeratins, respectively. These proteins are present in most epithelial cells and are, perhaps, the most divergent and atypical of all IF proteins. Class III IFs are formed by proteins expressed in different tissues: glial fibrillary acidic protein (GFAP), characteristic of astroglial cells; desmin, present in muscle cells; synemin, in certain muscles; peripherin, specific for the peripheral nervous system as well as for some central nervous system (CNS) neurons, and vimentin, present in all mesenchymal tissues. The pattern of vimentin expression differs from that of other IFs, since the protein appears transiently in a wide variety of cells during development. Classe IV IF proteins are formed by neurofilaments (NF) and internexin present in neurons and by nestin, present in precursors of the nervous system (NS) and muscles. The lamins, lining the inner surface of the nuclear membrane, represent the class V IF ubiquitously expressed throughout most cell types.Recently, new classes of IFs have been described: filensin and phakinin, named the beaded filaments, a novel class of IFs present in the lens (6) and, more recently, transitin, a novel IF protein that is transiently expressed by radial glia during CNS development. This protein is expressed by midline radial glia structures, by several axon commissures and by Bergmann glia of the developing cerebellum (7).

3. GFAP AND VIMENTIN: INTERMEDIATE FILAMENTS IN ASTROCYTES

One of the main hallmarks of developmental neurobiology is to understand the molecular mechanisms by which such cellular diversity is generated. Such diversification occurs at an early stage of development, especially by activation of sets of cell type-specific genes, which gives cells distinctive functions and morphological characteristics. Some of these cell-specific genes are the IF protein genes, which are regulated during cell development. The two major IF proteins of astrocytes are vimentin and GFAP. In the course of astrocyte development, a transition in the expression of IF protein genes is observed. Early during development, radial glia and immature astrocytes express mainly vimentin (5). Towards the end of gestation, a switch occurs whereby vimentin is progressively replaced by GFAP in differentiated astroglial cells (8, 9). At present, there is no consensus on the functional role of these IF proteins. The application of molecular genetic approaches to IF function has been providing some significant insights as well as raising new questions about the functional role of individual IF proteins.

3.1. Vimentin

Vimentin IFs are the only IF type found in a variety of cells including astrocytes, fibroblasts, endothelial cells, macrophages, neutrophils and lymphocytes (10). Functional analysis of the vimentin gene promoter has already been carried out and several negative and positive elements were identified within this region (11). Data obtained from vimentin knockout mice (-/-) demonstrated that those animals developed and reproduced without presenting an obvious new phenotype, thus heavily calling into question the biological function of vimentin (12). Several data, however, argue in favor of a relevant function for vimentin. Using the same vimentin (-/-) lineage as Colucci-Guyon *et al.* (12) found that GFAP filaments were also absent in certain glial cells that normally coexpress vimentin and GFAP such as the Bergmann glia and an astrocyte subpopulation of the corpus callosum. This was not due to the inability to express GFAP. Transfection of cultured vimentin -/- astrocytes with a vimentin cDNA restores the vimentin-GFAP filament network, suggesting that in these cells vimentin might be required for coassembly with GFAP filaments (13).

Reactive gliosis is a prominent result of many types of insult to the central nervous system (CNS) and leads to the formation of glial scar that impedes the regeneration of axons. The intermediate filament protein vimentin is found in pathology of the CNS, mainly in the vicinity of injuries to the CNS. In the present study some authors investigated the role of vimentin in the formation of glial scars *in vitro* and *in vivo* by using immunohistochemistry, Western blot analysis, and *in situ* hybridization. *In vitro* experiments showed that the intensity of immunofluorescent labeling for vimentin and glial fibrillary acidic protein (GFAP) was consistently decreased in astrocytes after transfection with a retrovirus carrying antisense complementary DNA (cDNA) for vimentin. Transfection also inhibited the growth of astrocytes and decreased the expression of vimentin mRNA. *In vivo* studies demonstrated that transfection with the retrovirus carrying the antisense cDNA vimentin inhibited the upregulation of vimentin and GFAP in stab wounds in rat cerebrum. These results suggest that

vimentin may play a key role in the formation of glial scars in the CNS.

Moreover, vimentin appears to accompany the formation of glial scars. Vimentin may stabilize the formation of GFAP-type IF in some reactive astrocytes, and its expression may be required for the formation of GFAP in these cells (13). In normal adult CNS, vimentin is not expressed in astrocytes, but only in some specialized glial cells such as those of Bergmann glia and radial glia, and ependymal cells. These findings suggest that vimentin may take part in the formation of glial scar, and that there may be a relationship between the expression of GFAP and that of vimentin. During the formation of GFAP networks in some reactive astrocytes, vimentin may act as a cytoskeleton associated protein (14). At early stages of CNS development, IF in radial glia and immature astrocytes are composed of vimentin (15). Subsequently, at about the time of birth, a transition from vimentin to GFAP takes place; vimentin disappears and is progressively replaced by GFAP in differentiated astroglial cells, which transiently coexpress these two proteins (13). The transient expression of vimentin observed in the present study has also been observed immunocytochemically in most models of gliosis (16, 17, 18). In the normal adult rodent brain, vimentin expression is restricted to specialized glia such as ependymal cells, Bergmann glia of the cerebellum, and Schwann cells, which has led to the suggestion that vimentin may be a more specific marker of gliosis than is GFAP (17, 19). Galou (13) founds that the astrocytes in the immediate vicinity of stab wounds expressed considerable GFAP. However, these cells did not express GFAP after the vimentin gene had been knocked out, whereas in wild-type mice the cells not only expressed vimentin but also expressed GFAP. These results suggest that the expression of GFAP in these astrocytes depends on the expression of vimentin, and that changes in the expression of vimentin affect the expression of GFAP. In summary the authors Lin and Kai found that the expression of vimentin and GFAP increased markedly after injury to CNS, and that restricting vimentin decreased the expression both of vimentin and GFAP, as well as formation of glial scar. In addition the authors therefore believe that vimentin may play an important role in reactive gliosis and the formation of glial scar. Accordingly, we suggest that manipulating the expression of vimentin may control reactive gliosis and provide an environment that favours the regeneration of injured axons.

3.2. Glial fibrillary acidic protein

Initially isolated from multiple sclerosis plaques, GFAP has been widely recognized as an astrocyte differentiation marker, constituting the major IF protein of mature astrocyte (20).

The developmental schedule of GFAP expression is not known in detail. In the mouse CNS, GFAP expression has been first detected at the end of gestation (9).Transcriptional studies demonstrated that GFAP mRNA increases between birth and day 15 and then decreases until day 55 (21). After reaching a plateau lasting into the second year of adult life, GFAP mRNA and protein levels tend to increase again in some regions such as the hippocampus, striatum and cortex (22). This usually corresponds to the increase of reactive astrocytes. Such increase during senescence is one of the most generalized markers for brain aging.

Insights into the role of GFAP have only recently emerged with reports on subtle abnormalities in GFAP-deficient-mice (23, 24).

Recently, Pekny *et al.* (25) have shown that primary cultures of GFAP-/- astrocytes exhibited an increased final cell saturation density. Those results led the authors to speculate that the loss of GFAP expression observed focally in a proportion of human malignant gliomas might reflect tumour progression to a more rapidly growing and malignant phenotype.

Some data indicate a novel concept that GFAP might play an important role in the control of neurological disease. Liedtke *et al.* (26) analyzed the astroglial response in GFAP-/- mice with experimental autoimmune encephalomyelitis, a model for multiple sclerosis. GFAP-/- astrocytes presented a disorganized cytoarchitecture due to irregular spacing and a decreased number of hemidesmosomes.

Although there is still some controversy about GFAP function in brain physiology and pathology, a large amount of evidence has been accumulating in the past few years in favour of an active and relevant role for this structural intermediate filament protein in brain development.

4. EFFECT OF GROWTH FACTORS AND HORMONES ON ASTROGLIAL CELL DIFFERENTIATION

GFAP synthesis is considered an important element of the developmental program of astrocyte differentiation and is part of the reactive response to almost any CNS injury.

Thus by determining how transcription of the GFAP gene is controlled in these circumstances, insights should be obtained into the more general mechanisms of CNS development and disease. Indeed several putative growth factor binding domains have already been identified in the GFAP gene promoter region.

Thyroid hormones are known to be important for normal brain development. Experiments *in vivo* have shown that neonatal treatment of rats by subcutaneous injections of T3 affects the development of astroglia in forebrain and hippocampus, accelerating the transition from vimentin-positive to GFAP positive cells in both brain regions (27).

In fact, the authors have reported that T3 treatment induces cortical astrocytes which presented a flat morphology *in vitro* to become process-bearing cells (28, 29).

Unlike cortical astrocytes, cerebellar astrocytes did not alter their morphology in response to T3. Rather, those astrocytes proliferate due to hormonal treatment. T3 did not increase GFAP synthesis in cerebellar astrocytes *in vitro* although it promoted alteration in GFAP organization. T3 can induce GFAP synthesis and astrocyte differentiation by promoting synthesis and secretion of growth factors which themselves can act autocrinally on astrocytic cells (28).

There is evidence that glucocorticoids inhibit GFAP expression both at the mRNA or protein level *in vivo* (30, 31). However, there are contradictory results regarding the effects of glucocorticoids on GFAP expression *in vitro*. Astrocyte cultures from neonatal rats generally show an increase in GFAP expression when treated with corticosteroid (32).

Glucocorticoid response element has been identified in the GFAP gene promoter with a putative functional significance.

Previous studies have demonstrated that estradiol promotes differentiation of glial cells from a flat morphology to a process-bearing one. Such morphological change is followed by a redistribution of GFAP in hypothalamic astrocytes *in vitro* and is dependent on the presence of neuronal cells (33). In our recent research (34) we investigated the interactions between growth factors (GFs) and dexamethasone (DEX) on cytoskeletal proteins GFAP and vimentin (VIM) expression under different experimental conditions. Condition I: 24h pretreatment with bFGF, subsequent 72h switching in serum-free medium (SFM) and final addition of GFs, alone or by two in the last 24h, after a prolonged (60 h) DEX treatment. Condition II: 36h pretreatment with DEX (with bFGF in the last 24h), followed by SFM for 60h and final addition for 24h with growth factors alone or two of them together.

Western blot analysis data showed a marked GFAP expression in cultures submitted to Condition I comparing results to untreated or treated controls. VIM expression was instead significantly reduced after GFs addition in the last 24 h of 60h DEX treatment, respect to control DEX-pretreated ones. Referring data to untreated controls, VIM expression was significantly enhanced after GFs addition. GFAP showed also a significant increase in astrocytes submitted to Condition II, respect to untreated or treated control cultures. VIM expression was up and down regulated under Condition II**.** (34)

Another study (35) was designed to assess the interactions between growth factors and dexamethasone (DEX) on cytoskeletal proteins (GFAP and vimentin) expression in 25 days *in vitro* (DIV) astrocyte cultures. An increase in GFAP and vimentin expression was observed after 12h pretreatment with bFGF and subsequent treatment for 60h with DEX. GFAP immunoreactivity was decreased after 24h progression growth factors (EGF, IGF-I and INS) addition, when compared to control 36 h DEX and bFGF-pretreated cultures for the last 12h. Vimentin immunoreactivity was decreased after 12h bFGF pretreatment and subsequent 60h DEX addition in astrocyte cultures compared to 12h bFGF-pretreated ones. Pretreatment for 36h with DEX plus bFGF in the last 12h and subsequent treatment for 24h with DMEM (Dulbecco's modified Eagle medium; DMEM) + BSA (bovine serum albumine) (harvesting), or with progression growth factors (EGF, IGF-I or INS) alone or two of them together, stimulated GFAP expression, compared to untreated controls. Immunochemical analysis of the mitogen-activated protein kinase ERK2 suggests an involvement of this enzyme in the control of GFAP expression. The above findings support the view of an interactive and complex dialogue between growth factors and glucocorticoids during astroglial cell proliferation and maturation in culture. (35).

In our previous researches, (36) data on GFAP Western blot densitometry in astrocyte cultures pretreated with bFGF for 12h, and subsequently treated with estradiol (E_2) for 24h and growth factors in the last 12h, have been obtained. Pre-treatment for 12h with bFGF followed by exposure to E_2 for 24h remarkably increased GFAP immunoreactivity.This effect was countered by addition of EGF or IGF-I or INS in the last 12h of the 24h E_2 treatment. Densitometric analysis of vimentin Western blots has shown a significant increase of protein expression in astrocyte cultures pretreated for 12h with bFGF and then treated for 24h with E_2. Addition of EGF, IGF-I or INS in the last 12h of the 24h exposure to E_2 remarkably decreased vimentin expression compared to control situation. Progression growth factors added in the last 12h of the 24h time of exposure to E_2 caused a noteworthy stimulation for IGF-I or INS and a weak decrease for EGF in comparison with untreated or/and pretreated controls respectively.

These findings demonstrate a synergistic effect evoked by the interaction between E_2 and growth factors during astroglial cell proliferation and differentiation in bFGF pretreated cultures. When progression growth factors were added in the presence of a differentiative factor such as E_2, GFAP and vimentin expression was markedly enhanced in comparison to untreated controls. On the contrary, expression of these astroglial markers was reduced compared to pretreated controls. These data confirm the occurrence of a modulation of cytoskeletal protein expression depending on growth factor-E_2 interaction during cell proliferation and differentiation in our *in vitro* experimental model (36).

5. GFAP EXPRESSION DURING NEURON-GLIA CROSS-TALK

Although some studies have postulated a requirement of GFAP for the formation of astrocytic processes, there is still some controversy about such mechanism. Pekny *et al.* (25) reported that GFAP-deficient astrocytes in the primary cerebellar cultures could form normal processes in response to neurons. Thus GFAP might not be a sole prerequisite for the formation of astrocytic processes.

Another remarkable example of the relevance of GFAP expression in neuron-glia interaction regards regeneration of axonal growth after CNS lesions. The GFAP-glial scar formed after NS lesion is considered to be a barrier to regeneration of axon growth.

GFAP synthesis inhibition relieved the blockage of neurite outgrowth that normally is observed after a lesion.

In contrast to this study, Wang *et al.* (37) did not find a correlation between the absence of GFAP and axonal outgrowth. They reported no increase in axon sprouting or long distance regeneration in the cortical spinal tract fibers of GFAP-/- mice.

Given the relevant role of GFAP during CNS development, as well as a factor in the reactive response to injury, understanding the mechanism of its modulation should be useful to elucidate some steps of NS physiology and pathology.

Several lines of evidence indicate that glia influence the growth, migration and differentiation of neurons but the effect of neuronal cells on astrocytes is far from being well understood. Increasing evidence has been accumulated indicating that neurons are modulators of astrocyte gene expression and differentiation (38, 39).

Recently, the authors demonstrated that neurons secrete brain region-specific soluble factors which induce GFAP gene promoter (38).

Furthermore, GFAP is differently modulated by distinct brain regions. It is conceivable that differences in growth factor binding ability of the GFAP gene promoter from different regions could account for such diverse modulation.

Taken together, those results emphasize the complexity of neuron-glia interaction during CNS development and suggest that neurons may modulate the GFAP gene promoter and induce the astrocytic differentiation program. These data argue in favour of the possibility that modulation of intermediate filaments such as GFAP in astrocytes by growth factors might be implicated in cell differentiation as well as in cell-cell interactions during CNS development.

6. NESTIN

Nestin is a class VI intermediate filament protein that was first identified in the progenitor cells that are found during the early developmental stages of the central and peripheral nervous systems (40). Subsequently, nestin was detected in progenitor cells of non-neuronal tissues, such as skeletal and cardiac muscle (41), tooth (42), testis (43), hair follicle cells (44), pancreas (45), oval cells in the liver (46) and mammary gland (47). Owing to its characteristic expression pattern, nestin generally is considered to be a marker of stem or progenitor cells. In the adult mammalian brain, nestin has been detected in stem and progenitor cells that reside in two neurogenic regions, the subventricular zones of the lateral ventricle (48) and the subgranular zone of the hippocampal dentate gyrus (49). Nestin has also been observed in non-germinal cells in the brain, such as reactive astrocytes (50), endothelial cells (51) and pericytes (52). There is a general agreement on the Nestin re-expression in reactive astrocytes, but its modalities differ among experimental animal species and between the latter and human material.

In comparison with GFAP, Nestin was poorly expressed in mature astrocytes and more expressed in developing reactive astrocytes, mainly in the cytoplasm, with a great variability, and much less in the processes. In the invading tumour, developing positive astrocytes were hardly distinguishable from tumour invading astrocytes that, interestingly, were much more Nestin- than GFAP-positive. In the deep tumour reactive astrocytes were no more visible. In reactive conditions, a hypertrophy of cellular processes with up-regulation of GFAP and Vimentin and re-expression of Nestin occur; a number of genes are involved with the cell functions of reactive astrocytes (53). Nestin and Vimentin would be the main IF of immature, whereas GFAP and Vimentin of the mature astrocytes (54). In the post-natal brain, GFAP and Vimentin would replace Nestin (55).

In normal rat brain, Nestin occurs in few astrocytes of the brain stem, whereas in reactive astrocytes it has been observed everywhere: in hippocampus by lesions with kainic acid, in hemispheres in experimental ischemia (56, 57) and in trauma where Nestin expression increases in time with GFAP (58). Nestin is expressed in neuroepithelial stem cells, radial glia cells and progenitor cells. It is down-regulated at the onset of GFAP expression during terminal differentiation to mature astrocytes. There are today numerous contributions showing that Nestin is re-expressed in reactive astrocytes where, as an embryonal feature of neuroepithelial cells, it would play a role in their plasticity. This Nestin re-expression seems today well documented by its demonstration following experimental hippocampal lesions (59), spinal cord injuries (60), global cerebral ischemia (61), mechanical cortical injury (62) and also in human pathology, such as in multiple sclerosis (63) or around brain tumours (64).

The various observations reported in the literature diverge, but not for the different interpretations given to the findings, because the general significance of Nestin re-expression is referred in any case to a kind of embryonal regression. They are based mainly on the assumption that reactive astrocytes follow the same steps known in the physiological development of astrocytes from stem cells, precursors and progenitors during embryogenesis.

Reactive astrocytes are supposed to have originated long before and have reached their final stage of maturation. GFAP definitely prevails upon Nestin decorating cytoplasm and every kind of processes, including the aspect of the fibrous gliosis, where Nestin is very poor and limited to the small cytoplasm

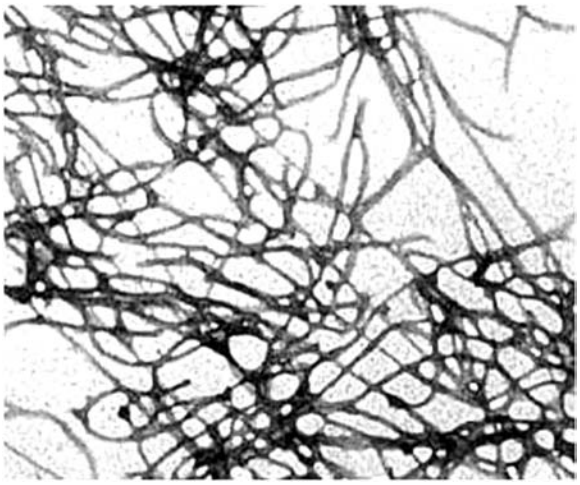

Figure 1. Vimentin filaments observed by negative staining and transmission electron microscopy.

The prevailing distribution of GFAP and Vimentin in the thin and long processes in comparison with Nestin is less evident in younger than in older reactive astrocytes, whereas the expression of Nestin in the cytoplasm is highly variable, probably as a consequence of their different durations. It is worth emphasizing that, in line with the above mentioned interpretation, reactive astrocytes with large bodies surrounding tumours can be either intensely positive for Nestin or almost negative, whereas their GFAP and Vimentin expression is more stable. In peri-tumoral tissue, reactive astrocytes can show mitosis and more frequently in Nestin than in GFAP or Vimentin-positive cells, probably for the more embryonal character indicated by Nestin occurrence. A recent paper (65) the authors found nestin-immunoreactive cell type in the adult rat cerebral cortex under normal conditions. The authors immunohistochemically analyzed which types of cells exhibit immunoreactivity for nestin, and identified these cells as microglia using a laser confocal microscope. Moreover, the authors checked the coexistence of another intermediate filament protein vimentin, which is a possible candidate for nestin-interacting partner. In addition, they identified nestin and vimentin- positive microglia in the adult rat cerebral cortex. Nestin-positive or vimentin-positive microglia have been previously observed in the brain when pathological conditions are present. Nestin-positive microglia were observed after a cortical injury in the rat brain (66) and after a neurodegeneration of dentate gyrus granule cells in the mouse brain (67). Vimentin-positive microglia have been observed in the rat brain after several stimuli, including in the facial nucleus after a facial nerve axotomy, in the hippocampal formation after an axonal degeneration due to entorhinal cortex lesions and in the substantia nigra after the injection of lipopolysaccharide, which is a potent immunostimulant (68). Vimentin-positive microglia have also been observed in the human brain during pathological conditions, such as amyotrophic lateral sclerosis, multiple sclerosis, Alzhèimer's disease, infarction and Creutzfeldt–Jakob disease. As these microglia were observed in brains when pathological conditions were present, they should have been in an activated state. These previous observations in conjunction with results obtained by these authors, suggest that nestin and vimentin are expressed in activated microglia as well as in the subset of microglia that are in a resting state.

In this study, the authors found that nestin is expressed in a subset of microglia that were non-germinal and terminally differentiated. The expression of nestin in differentiated cells has been previously reported in reactive astrocytes (60), endothelial cells (51), pericytes (52).In addition, Takamori *et al.*, have also reported co-localization between nestin and other intermediate filament proteins include, vimentin and desmin in the human fatal G6 myoblast cell line and vimentin in several cultured cell lines (69). While nestin can be co-assembled into intermediate filament networks in the presence of other intermediate filament proteins, it is unable to self-assemble in cultured cells *in vitro* (70) or in astrocytes and endothelial cells *in vivo*. In particular, nestin and vimentin are co-localized, and it suggests that nestin is co-assembled with vimentin to form intermediate filament networks in the microglia *in vivo*.Nestin, a currently used marker of neural stem cells, is transiently co-expressed with glial fibrillary acidic protein (GFAP) during development and is induced in reactive astrocytes following brain injury. Nestin expression has also been found in cultures of astroglial cells, but little is known about the fate and the mitotic activity of nestin-expressing cells in this *in vitro* model. A recent study Sergent-Tanguy *et al.*, (71) reveals a long-lasting expression of nestin in primary cultures of astroglial cells derived from the rat brain. Over 70% of the cells were nestin+ at 12 weeks, with a large majority co-expressing the GFAP astrocytic marker. Time-course analyses supported a transition from a nestin+/GFAP– to a nestin+/GFAP+ phenotype over time, which was further increased by cell cycle arrest.

These observations indicated that nestin+/GFAP– cells are actively engaged in mitotic activity, even after 2 weeks *in vitro*. Part of these cells might have retained properties of neural stem cells, insofar as 10% of cells in a primary culture of glial cells were able to generate neurospheres that gave rise to both neurons and astrocytes.

Further studies will be necessary to characterize fully the proliferating cells in primary cultures of glial cells, but the results obtained in a recent paper reveal a major contribution of the nestin+/GFAP– cells to the increase in the number of astrocytes, even though nestin+/GFAP+ cells proliferate also. These observations suggested a role of nestin+/GFAP+ cells as NSC or a contribution of these cells to the formation of glial scar. Interestingly, Ernst and Christie (72) recently showed that the expression of nestin did not predict an active state of proliferation. Indeed, nestin+/GFAP+ cells present in the unlesioned neocortex are not actively engaged in mitotic activity, although reactive astrocytes that express nestin in response to injury are mitotically active.

In previous studies Sergent-Tanguy *et al.*, have reported an increase in GFAP expression over time in culture and the ability of GFAP+ cells to proliferate in

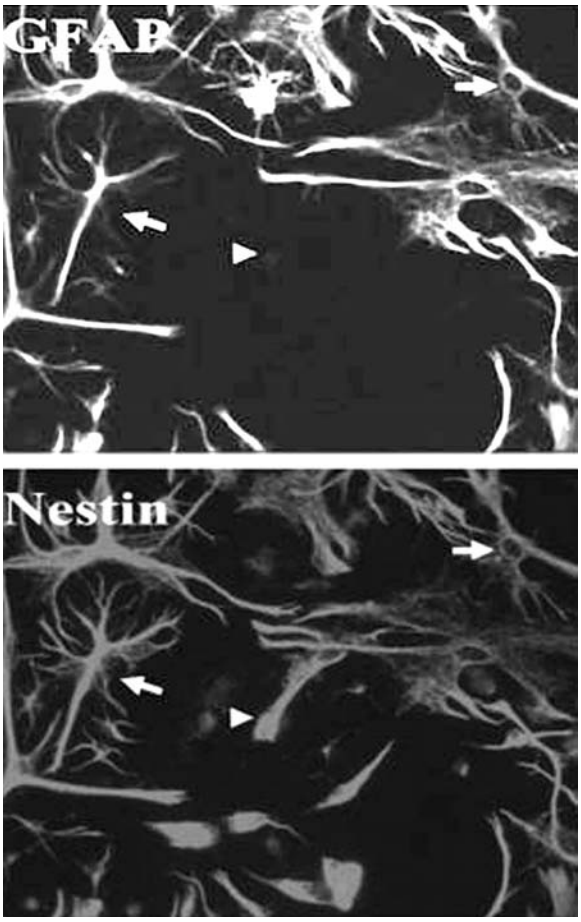

Figure 2. Immunocytochemistry. The cells were fixed with 4% PFA and sequentially immunostained with antibodies directed against GFAP and nestin. Arrows, nestin+GFAP+ cells; arrowhead, nestin+GFAP– cell

cultures of astroglial cells (73). In addition, these authors show a long-lasting expression of nestin in astroglial cell cultures derived from the rat brain, together with an increase in number of nestin+/GFAP+ cells over time.

The results also reveal that most cycling cells express nestin but not GFAP, suggesting a major contribution of nestin+/GFAP– cells to the increased number of astrocytes even after 15 days *in vitro*. Because of the long-lasting expression of nestin in primary cultures of rat glial cells, double-staining immunocytochemistry was performed to determine whether this IF was expressed by GFAP+ cells. The photos in Figure 2 (arrows) clearly show double-labeled cells, staining positive for nestin and GFAP at 2 weeks *in vitro*. Quantification with two-color flow cytometry indicated that 46.7% 6 2.3% of the cells were nestin+/GFAP+.

Very few cells (2.3% 6 0.2%) expressed only GFAP, whereas 23% 6 1.7% of the cells were nestin+/GFAP– (Figure 3). Later in the culture, a significant decrease in the number of nestin+/GFAP– cells was observed, together with an increase in the number of nestin–/GFAP+ cells (Figure 3), although the major changes concerned the nestin+/GFAP+ cell population, in that 79.4% 6 0.2% of the cells expressed both IFs at 8 weeks *in vitro*. However, in astrocyte monolayers derived from the mouse cerebral cortex and cultured for 10 days in serum-containing medium, more than 95% of the cells were found to express nestin (74). Here, we show an expression of nestin over 12 weeks in primary cultures of rat astroglial cells.

One hypothesis is that the lack of growth factor or inadequate cellular environment prevents the full differentiation of astrocytes *in vitro* (75). However, this long-lasting expression is reminiscent of that observed for vimentin, and, as previously shown in well differentiated cultures of astroglial cells (76), an expression of vimentin for up to 8 weeks was observed under our culture conditions (data not shown). Such long-lasting expression of vimentin and nestin may be due to the fact that astroglial cells in culture exhibit many features of reactive glial cells (77).

The exact biological significance of nestin reappearance and GFAP up-regulation in reactive astroglial cells is not yet known, but modifications in IF composition have been suggested to be important for the changes in cell shape that accompany astrogliosis.

The transient co-expression of nestin and GFAP during brain development (55) or after brain injury probably confers astroglial cells with particular properties that remain to be defined. Alternatively, the nestin+ / GFAP+ cells might have a lower rate of proliferation than the Nestin+/GFAP– cells. The low number of cell divisions in C6 cells over-expressing GFAP (78) supports such a possibility. In addition, GFAP+/nestin+ B cells located in the adult SVZ were reported as slowly proliferating cells, whereas type C cells that expressed nestin but not GFAP were described as rapidly dividing immature precursors (79). The presence of nestin+/GFAP– cells in astroglial cultures suggested the presence of cells that might have retained properties of NSCs, such as the capacity of proliferation upon treatment with mitogen and the possibility of generating neurons and astroglial cells as shown in mice (74). Here, Sergent-Tanguy et al., (71) show that about 10% of the cells in 2-week-old cultures generate neurospheres following exposure to FGF-2/EGF and that more than 80% of these neurospheres give rise to both astrocytes and neurons under differentiating conditions. Further studies will be necessary to characterize fully the nestin+/GFAP– cells present in primary cultures of astroglial cells, but the recent paper has revealed that over 75% of the cells still expressed nestin at 12 weeks in vitro. These authors have also provided the first evidence that nestin+ cells are mitotically active in standard conditions of astroglial cell cultures. Finally, Sergent-Tanguy et al., (71) showed that nestin+/GFAP– cells and nestin+/GFAP+ cells are both capable of proliferating in 2-week-old cultures, but the former contribute more to the increase in cell number, insofar as most cycling cells express nestin but not GFAP

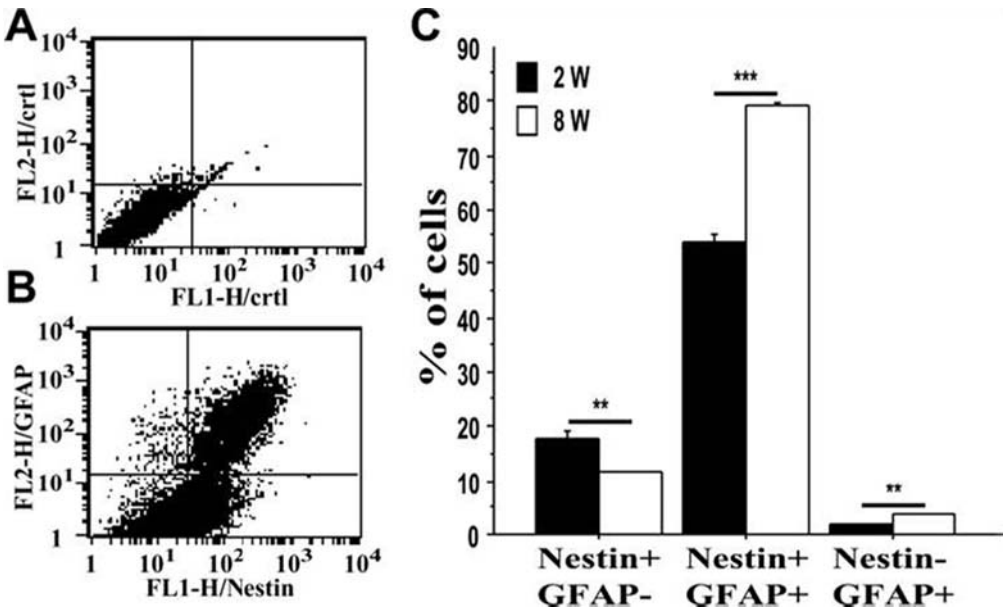

Figure 3. Percentage of nestin+ and GFAP+ cells in primary cultures of astroglial cells. The cells were grown for 2 or 8 weeks in serum-supplemented medium, prior to detachment with trypsin, fixation, and sequential labeling with antibodies directed against nestin (FL1-H) and GFAP (FL2-H). The number of single- or double-immunopositive cells was estimated by flow cytometry (B) and plotted (C). The background level was estimated with secondary antibodies (A). Values are means 6 SEM, n ¼ 3.

7. S-100B

S100B is a member of the S100 family. This family of proteins was termed "S100" because it was soluble in 100% saturated ammonium sulfate solution. S100B is an acidic protein with a molecular weight of 21 kDA existing as a homodimer consisting of two beta subunits. The two monomers are configured in a twofold axis of rotation and are held together by disulfide bonds (80). The disulfide-linked form of S100B appears to be required for fully functional neurotrophic action, while S100B mutants lacking one or both of the cysteine residues induce activation response in glia but not in neurons (81). S100B is a $Ca2^{+}$-binding protein of the EF-hand type (helix-loop-helix) with 4 Ca-binding sites (82). It also binds copper at 4 binding sites and Zn^{2+} at 6-8 binding sites (82), and such binding influences the Ca-binding capacity of the protein.

This protein has been implicated in the Ca2+-dependent regulation of a variety of intracellular functions such as protein phosphorylation, enzyme activities, cell proliferation and differentiation, dynamics of cytoskeleton constituents, structural organization of membranes, intracellular Ca^{2+} homeostasis, inflammation, and protection from oxidative cell damage.

The gene encoding S100B in humans is located on chromosome 21q22.3 at a distance of 100–140 kb from the chromosome terminus (83). S100B is produced primarily by astrocytes and exerts autocrine and paracrine effects on glia, neurons, and microglia (84). On the other hand, elevations of S100B levels in blood or cerebrospinal fluid have been observed in patients with Alzheimer's disease, Down's syndrome, amyotrophic lateral sclerosis, multiple sclerosis, schizophrenia, depression, cerebral stroke and traumatic brain injury, and the levels have reached micromol/L-order at focal regions. It is thought to be released from glial cells via a mechanism similar to that governing the secretion or release of other factors such as ciliary neurotrophic factor, interleukin-1_ and 1_, or human endothelial growth factor (85).

It has been documented that the excessive S100B promotes the expression of inducible nitric oxide synthase or pro-inflammatory cytokines and exhibits detrimental effects on neurons. On studies using some animal models of the cerebral stroke or Alzheimer's disease, it is suggested that the excessive S100B produced by activated astrocytes precedes neurodegenerations. Authors discussed the relationship between neurological disorders and the S100B.

Also, insights have been gained regarding the interaction between S100B and the cerebral immune system, and the regulation of S100B activity through serotoninergic transmission. Secreted glial S100B exerts trophic or toxic effects depending on its concentration. At nanomolar concentrations, S100B stimulates neurite outgrowth and enhances survival of neurons during development. In contrast, micromolar levels of extracellular S100B *in vitro* stimulate the expression of proinflammatory cytokines and induce apoptosis. In animal studies, changes in the cerebral concentration of S100B cause behavioural disturbances and cognitive deficits. In humans, increased S100B has been detected with various clinical conditions. Brain trauma and ischemia is associated with increased S100B concentrations, probably due to the destruction of astrocytes. In neurodegenerative, inflammatory and

psychiatric diseases, increased S100B levels may be caused by secreted S100B or release from damaged astrocytes.

In an interesting review (86) findings on S100B regarding human brain damage and neurodegeneration have been summarized. Findings from *in vitro* and *in vivo* animal experiments relevant for human neurodegenerative diseases and brain damage are reviewed together with the results of studies on traumatic, ischemic, and inflammatory brain damage as well as neurodegenerative and psychiatric disorders.

During the last decade, increasing efforts have been made to understand the role of S100B in astrocytes, neurons, and microglia cells. *In vitro* experiments in cell cultures and *in vivo* investigations in animals have made major contributions towards understanding the significance of S100B in the brain. With improving knowledge about S100B, clinical studies in various diseases and conditions have been undertaken to facilitate the application of knowledge from basic scientific experiments to clinically relevant problems in humans.

On the basis of the findings published until now, these authors now propose the following suggestions for further research, taking neurodegenerative disorders and brain damage into account in particular (86).

Important contributions have come from behavioural animal experiments involving various conditions with increased or decreased S100B levels in the brain. The fact that knockout and transgenic mice with adjustable S100B production are now available should further stimulate research to improve the characterization of typical behavioural patterns. Experimental as well as natural environmental conditions should be tested. In addition, investigations on S100B in animal models for various neurologic and psychiatric disorders, e.g., Alzheimer's disease, would also broaden our view. These experiments would help to characterize the functional role of S100B *in vivo*.

In particular, Alzheimer's disease is by far the most extensively studied neurodegenerative disorder concerning S100B pathology. However, there are still many gaps that need to be filled in order to prove Mrak and Griffin's (87) plausible hypothesis. Interactions between S100B and the immunocompetent structures of the brain should receive special attention. This could provide additional insight into the pathophysiology of cerebral inflammatory disorders as well.

Regarding psychiatric disorders, a number of interesting preliminary results have been acquired. A follow-up of this subject might offer an opportunity to gain new insights into the pathogenesis of these disorders and to understand the metabolic processes involved in the reduction of dendrites and synapses leading to the pruning of neurons in schizophrenia and affective psychoses. Furthermore, detailed clinical characterizations of patient samples are required in order to identify subgroups of patients with reproducible changes in S100B concentrations.

8. CONCLUSIONS

In conclusion, the interaction between neuron and glial cells is essential to the harmonious development of the nervous system. Such interactions are based on junctional communication or on growth factors secreted by both cells which can mutually act on the metabolic patways or more directly on gene activation, thus modulating cell fate. In addition, the prevailing distribution of GFAP and Vimentin in the thin and long processes in comparison with Nestin is less evident in younger than in older reactive astrocytes, whereas the expression of Nestin in the cytoplasms is highly variable, probably as a consequence of their different durations. Finally, such glial biomarkers will help us to understand the more general mechanisms involved in CNS development and can open new perspectives for the control of the neurologic diseases.

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Abbreviations: GFAP: glial fibrillary acidic protein, DEX: dexametasone, E2: estradiol, EGF: epidermal growth factor, bFGF: basic fibroblast growth factor, INS: insulin, IGF-I: insulin like growth factor-I, MF: microfilaments, MT: microtubules, IF: intermediate filaments, NS: nervous system, CNS: central nervous system, NF: neurofilaments, cDNA: complementary DNA

Key Words: Astrocytes, intermediate filaments, GFAP, Vimentin, Nestin, S-100, Review

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