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Bis Is Involved in Glial Differentiation of P19 Cells Induced by Retinoic Acid

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Previous observations suggest that Bis, a Bcl-2-binding protein, may play a role the neuronal and glial differentiation in vivo. To examine this further, we investigated Bis expression during the in vitro differentiation of P19 embryonic carcinoma cells induced by retinoic acid (RA). Western blotting and RT-PCR assays showed that Bis expression was temporarily decreased during the free floating stage and then began to increase on day 6 after the induction of differentiation. Double immunostaining indicated that Bis-expressing cells do not express several markers of differentiation, including NeuN, MAP-2 and Tuj-1. However, some of the Bis-expressing cells also were stained with GFAP-antibodies, indicating that Bis is involved glial differentiation. Using an shRNA strategy, we developed bis-knock down P19 cells and compared them with control P19 cells for the expression of NeuroD, Mash-1 and GFAP during RA-induced differentiation. Among these, only GFAP induction was significantly attenuated in P19-dnbis cells and the population showing GFAP immunoreactivity was also decreased. It is noteworthy that distribution of mature neurons and migrating neurons was disorganized, and the close association of migrating neuroblasts with astrocytes was not observed in P19-dnbis cells. These results suggest that Bis is involved in the migration-inducing activity of glial cells.

Key Words: Bis, GFAP, Gliogenesis, Neuronal differentiation, P19

INTRODUCTION

Bcl-2 interacting death suppressor (Bis) has been identified as Bcl-2-binding protein (Lee et al., 1999). It has also been reported that Bis binds to Hsp70 or phospholipase Cγ, referred to as Bag3 or CAIR-1, respectively (Takayama and Reed, 1999; Doong et al., 2000). A growing body of evidence indicates that Bis may plan the role of a survival- or stress-related protein. An elevated expression of Bis has been reported in several types of cancer, including pancreatic cancer (Liao et al., 2001), thyroid carcinomas (Chiappetta et al., 2007) and certain forms of leukemia (Bonelli et al., 2004). In addition to these findings, it has been reported that the down-regulation of Bis expression sensitizes leukemia cells or colon cancer cells to apoptosis, several chemotherapeutic agents or 4-hydroxynonenal (Romano et al., 2003; Jacobs and Marnett, 2009) suggesting that the regulatory activity of Bis in cell death is one of its physiological functions, as demonstrated in our previous in vitro study (Lee et al., 1999). The expression of Bis has been reported to increase upon exposure to various stressful stimuli, such as heat shock, the presence of heavy metals and proteasome inhibitors (Pagliuca et al., 2003). Oxidative stress induced by transient ischemia or light has also been reported to induce Bis expression in reactive astrocytes or the retina, respectively, in animal disease models (Lee et al., 2002; Chen et al., 2004). Although the physiological significance of Bis induction that occurs under conditions of stress is not clear, Bis is generally thought to be involved in the control of the protein quality, as evidenced by the findings that the interaction of Bis and HspB8 lead to an increased rate of degradation of Htt43Q, aggregation-prone protein, by inducing macroautophagy (Carra et al., 2008).

Bis is expressed in a wide variety of tissues, including skeletal muscle, heart, kidney and thymus (Lee et al., 1999). The expression of Bis is observed transiently in the developing brain in cortical and hippocampal neurons during the late prenatal and early postnatal weeks, this disappears by the end of the second postnatal week. In addition, Bis is expressed in the meshwork of glial fibrillary acidic protein (GFAP)-positive astrocytes: i.e., glial tubes, in the rostral migratory stream (RMS) and subventricular zone (SVZ) of the lateral ventricle in the developing and adult forebrain (Park et al., 2003; Choi et al., 2006), and

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ABBREVIATIONS: RA, retinoic acid; GFAP, glial fibrillary acidic protein.

in radial glial cells in the developing CNS (Choi et al., 2009, in press). Thus, Bis may contribute to developmental processes, including the differentiation and maturation of neurons in the developing rat brain, and may also be associated with glial cells.

P19 embryonic carcinoma cells are a pluripotent cell line derived from mouse teratomas. P19 cells can be induced to differentiate into neurons, glia and fibroblast-like cells by treatment with all-trans retinoic acid (ATRA) (Jones-Villeneuve et al., 1982; Jones-Villeneuve et al., 1983). In this study, to investigate the role of Bis in the neuronal differentiation, we examined Bis expression during RA-induced differentiation of P19 cells. In addition, the effect of the down-regulation of Bis on the differentiation of P19 cells into neuronal or glial cells upon RA exposure was also investigated.

METHODS

Cell culture and neuronal differentiation

P19 embryonic carcinoma cells were cultured in DMEM (Thermo Scientific, Waltham, MA) with 10% FBS (Thermo Scientific), 100 units/ml of penicillin and 100 μg/ml of streptomycin in humidified incubator supplemented with 5% CO_2. In a typical experiment for the neuronal differentiation of P19 cells, 1×10^5 cells/ml cells were incubated in culture medium in the presence of 0.5 μM all-trans retinoic acid (RA, Calbiochem, La Jolla, CA) in non-adhesive bacteriological grade Petri dishes for 4 days. After 4 days of culture, the resulting cell aggregates were transferred to tissue culture dishes. The culture medium was then changed to culture medium that was free of RA and replenished every 2 days for 15 days.

Western blot analysis

The cells were lysed in RIPA (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 nM Tris-HCl) with 1 μM PMSF. The protein concentration was determined using a BCA protein assay reagent (Thermo Scientific, Waltham, MA). Equal amounts of protein were separated by SDS-PAGE and then transferred onto a PVDT membrane. The membrane was blocked with 0.1% TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) containing 5% non-fat dried milk for 1 hr and incubated with primary antibodies, after which, it was incubated with HRP conjugated IgG secondary antibody. In addition to anti Bis (1 : 10,000, Lee et al., 1999), anti GFAP (1 : 1,000, Chemicon, Temecula, CA) and anti GAPDH (1 : 100, Santacruz, Santa Cruz, CA) antibodies were also used as primary antibodies. Blots were detected using the Pierce ECL western blotting substrate (Theremo Scientific, Waltham, MA).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using RNA ZolB (Tel-Test Inc., Friendswood, TX) at the indicated days after RA treatment. The cDNA was then synthesized with a random hexamer primer and reverse transcriptase (Fermentas, Ontario, Canada). For the quantization of bis mRNA, real-time PCR was performed with IQ5 (Bio-Rad, Hercules, CA) using IQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Target mRNA values were normalized by GAPDH expression. The primers used in this study were as follows: bis forward 5′-tacatgagcagaatatcacccggc-3′, bis reverse 5′-ccagcagcctgtataccacaagat-3′, GAPDH forward 5′-ggtgtgaacggatttggccgtatt-3′, GAPDH reverse 5′-ggccttgactgtgccgttgaattt-3′. The expression of neuronal markers and a glial marker after the induction of differentiation in P19-con and P19-dnbis cells was examined by PCR amplification. The used primers were as follows: NeuroD forward 5′-tcaaccctcggactttcttg-3′, NeuroD reverse 5′-cattaagctgggcactcatg-3′ (Hong et al., 2008), Mash-1 forward 5′-caagttggtcaacctgggtt-3′, Mash-1 reverse 5′-gctcttgttcctctgggcta-3′, GFAP forward 5′-tttctcctttgtctcgaatga-3′, GFAP reverse 5′-ggtttcatcttggagcttct-3′ (Jing et al., 2009), GAPDH forward 5′-ggtgtgaacggatttggccgtatt-3′, GAPDH reverse 5′-ggccttgactgtgccgttgaattt-3′.

Bis knock-down using short hairpin RNA (shRNA) expression vector

To generate P19 cells in which Bis expression is constitutively down-regulated, a bis-specific shRNA expression vector was prepared using shRNA expression vector pSuper.puro (OligoEngine, Seattle, WA). Oligonucleotides specific for mbis were designed as follows: mbis sense (5'-gatccccaaggttcagaccatcttggaattcaagagattccaagatggtctgaacctttttttggaaa-3') and mbis antisense (5'-agctttccaaaaaaaggttcagaccatcttggaatctcttgaattccaagatggtctgaaccttggg-3'). The annealed double stranded oligonucleotide was ligated into the BglII and HindIII sites of pSuper.puro vector (pSuper.puro-sibis). P19 cells were transfected with the pSuper.puro-sibis or pSuper.puro vector using Lipofectamin2000 (Invitrogen, Carlsbad, CA) and selected against 2 μg/ml of puromycin for 10 days. Among the resulting 12 clones, two, which showed a significant reduction in Bis expression, were used in further experiments. The expression levels for the markers of neuronal development and GFAP were found to be essentially similar in the two clones. The results from one clone (P19-dnbis) are presented in our experiments with control P19 (P19-con) cells.

Immunocytochemistry

The cells were cultured on a cover glass, as described above (Fisher Scientific, Pittsburgh, PA). For fixation, cells were treated with 4% paraformaldehyde for 30 min and washed three times 5 min each with PBS. The fixed cells were treated for 10 min with 0.25% Triton X-100 in PBS for permeabilization. After washing, the cells were blocked for 30 min by treatment with 10% goat serum (GBI Inc., Mukilteo, WA) and then incubated with anti Bis (1 : 1,000, Lee et al., 1999), anti NeuN (1 : 1,000, Chemicon, Temecula, CA), anti MAP-2 (1 : 500, Chemicon, Temecula, CA), anti TuJ1 (1 : 500, Sigma-Aldrich, St. Louis, MO) and anti GFAP (1 : 1,000, Chemicon, Temecula, CA) antibodies. The cells were then incubated anti mouse-FITC (Invitrogen, Carlsbad, CA) and anti rabbit-Cy3 (Jackson, West Grove, PA) conjugated IgG antibodies.

RESULTS

Bis is increased during RA-induced differentiation of P19 cells

P19 cells were induced to undergo neuroectodermal dif-

ferentiation by culturing in Petri dishes in the presence of RA for 4 days, followed by incubation in culture dishes in the absence of RA for an additional 11 days. As shown in Fig. 1A, the major population of cells at day 8 had round cell bodies with long processes, indicating that they had undergone neuronal differentiation. A western blotting assay indicated that the expression of Bis was significantly decreased at day 4, at which time, numerous cellular aggregates were present, which continued 2 days after replating, i.e., on day 6. The significant increase in Bis levels was observed at day 9, and was maintained until day 15. Consistent with results of immunoblotting, Bis expression was not found in the cells of central embryonic bodies but was detected in surrounding cells by immunocytochemistry at day 12 after the induction of differentiation with RA (data not shown). The reduced expression of Bis at day 4 is not likely due to a loss of adhesion, based on that a suspension culture of P19 cells in a Petri dish without RA showed no difference in Bis levels (data not shown). In a quantitative analysis of the relative mRNA levels of Bis, measured by means of a real-time RT-PCR assay, the expression pattern was similar to that of Bis protein levels, indicating that the fluctuation in Bis levels during the RA-induced differentiation of P19 cells appears to be due to an alteration in the extent of transcription of bis mRNA. To characterize Bis expressing cells in differentiated P19 cells, induced by RA, we performed double labeling using markers for neural differentiation, including Tuj1, MAP-2 and NeuN and GFAP, an astroglial marker. As shown in Fig. 1C, Bis expressing cells do not express markers for differentiating and post mitotic neurons (Tuj-1 and MAP-2) and a more mature neuronal marker (NeuN), whereas some, not all, Bis-expressing cells co-express GFAP. Furthermore, the expression of Bis was stronger in Bis/GFAP double-labeled cells than in cells expressing only Bis. Since not all GFAP-positive cells express Bis, Bis seems to have a close relation to some function exerted by a subpopulation of glial cells.

GFAP induction was decreased by bis knock-down after RA-induced differentiation in P19 cells

To further study the function of Bis in the neuronal and glial differentiation of P19 cells, Bis expression was constitutively down-regulated using a bis-specific shRNA expression vector (P19-dnbis). P19-dnbis cells and control P19 (P19-con) cells were induced to differentiate by treatment with retinoic acid and mRNA and protein extracts were pre-

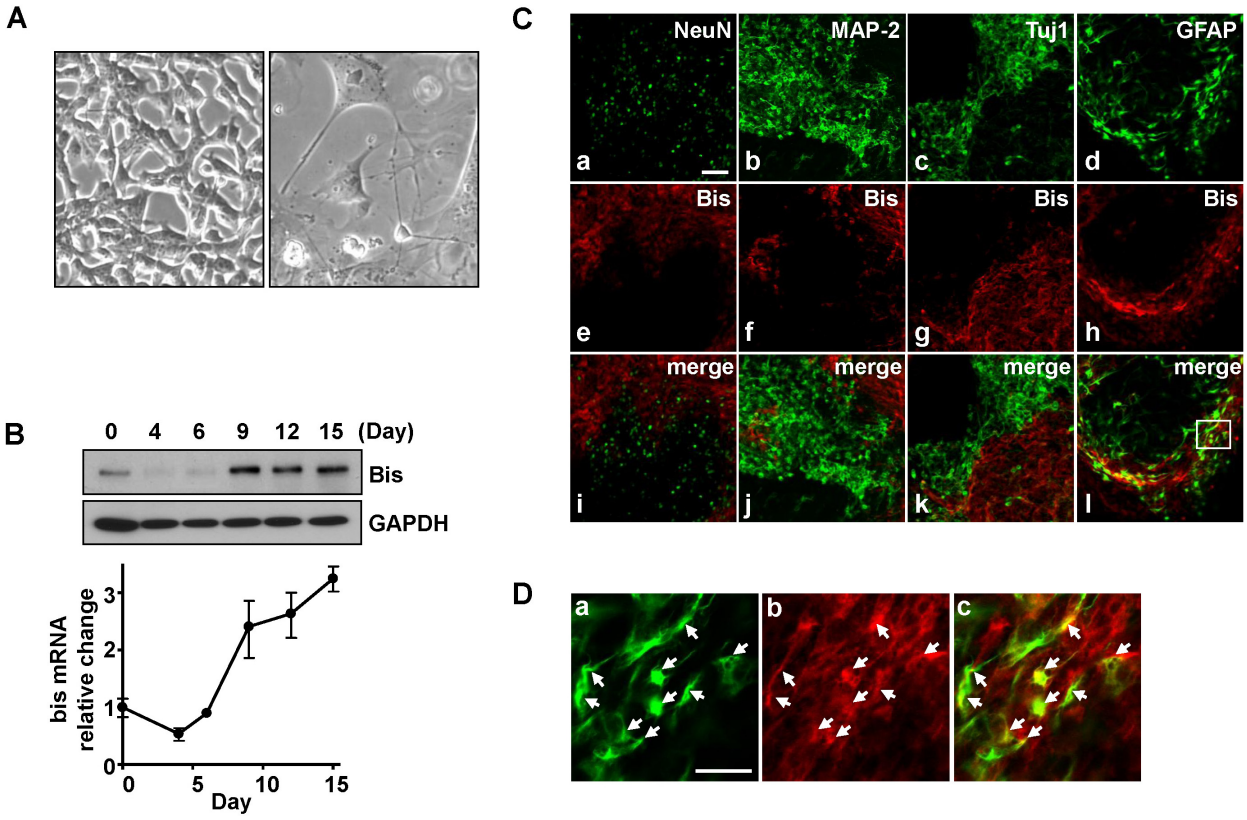

Fig. 1. Expression of Bis in GFAP-positive glia cells during RA-induced differentiation in P19 cells. (A) Light microscopic images of P19 cells before (left) and after (right) the induction of neuronal differentiation (day 12). (B) Change in Bis protein levels during the RA-induced differentiation of P19 cells was examined by immunoblotting (upper panel). The expression of GAPDH is shown as a loading control. The relative expression of bis mRNA levels was determined by real-time RT-PCR after normalizing to endogenous GAPDH control (lower graph). The value at day 0 was arbitrarily designated as 1.0. (C) Confocal laser microscopic imaging of immunofluorescence for Bis (e−h) and one of the marker antibodies for NeuN (a), MAP-2 (b), Tuj-1 (c) and GFAP (d) at day 12 after the induction of neuronal differentiation. (i−l) Superimposed images of FITC (a−d) and Cy3 (e−h). Scale bar, 50 μm. (D) Higher magnification of the boxed area in (Cl). Most GFAP labeled cells were immunoreactive for Bis (arrows in panel a−c). Scale bar, 20 μm.

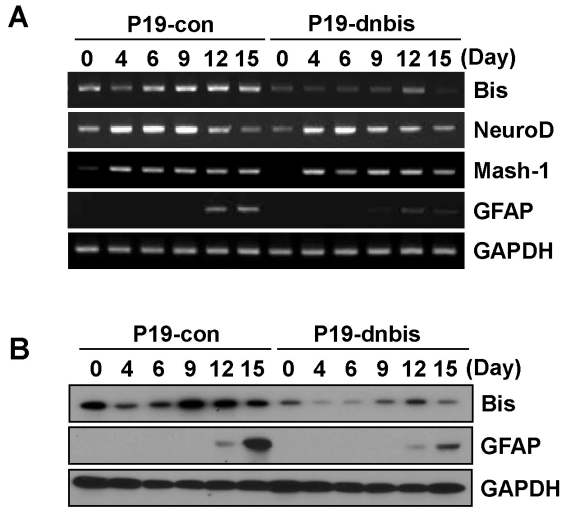

Fig. 2. Bis-knock down decreased the induction of GFAP in P19 cells after RA-induced differentiation. (A) Change in mRNA levels of Bis, NeuroD, Mash-1 and GFAP during RA-induced differentiation was examined by RT- PCR in P19-con and P19-dnbis cells. Note that GFAP induction was significantly decreased in P19-dnbis cells. (B) The expression of protein levels of Bis and GFAP was determined by immunoblotting in P19-con and P19-dnbis cells after the induction of differentiation with RA.

pared at the indicated days for analysis of the expression of neuronal or glial markers. As shown in Fig. 2A, bis mRNA expression was significantly attenuated in P19-dnbis cells after RA treatment as well as at basal levels. The induction of mRNA expression levels of neurogenic regulatory genes, such as NeuroD and Mash-1 was evident starting on day 4 and were maintained up to day 15 with similar expression patterns in P19-con and P19-dnbis cells, indicating that neuronal differentiation was not influenced by the down-regulation of Bis. The mRNA for GFAP was detected at day12 in P19-con cells, as previously reported (Santiago et al., 2005), which was notably suppressed in P19-dnbis cells. In addition to the mRNA levels, GFAP protein levels were also decreased considerably in P19-dnbis cells (Fig. 2). Collectively, these results suggest that Bis is involved in gliogenesis or glial differentiation, consistent with the partial co-localization of Bis and GFAP.

Bis suppression resulted in an impairment of glial differentiation and neuronal spreading in P19 cells after RA-induced differentiation

Although the expression of neuronal markers did not appear to be regulated by Bis, the expansion of differentiated cells from embryonic bodies was noticeably attenuated in P19-dnbis cells during RA-induced differentiation, as evidenced by the MTT staining of viable cells after re-plating

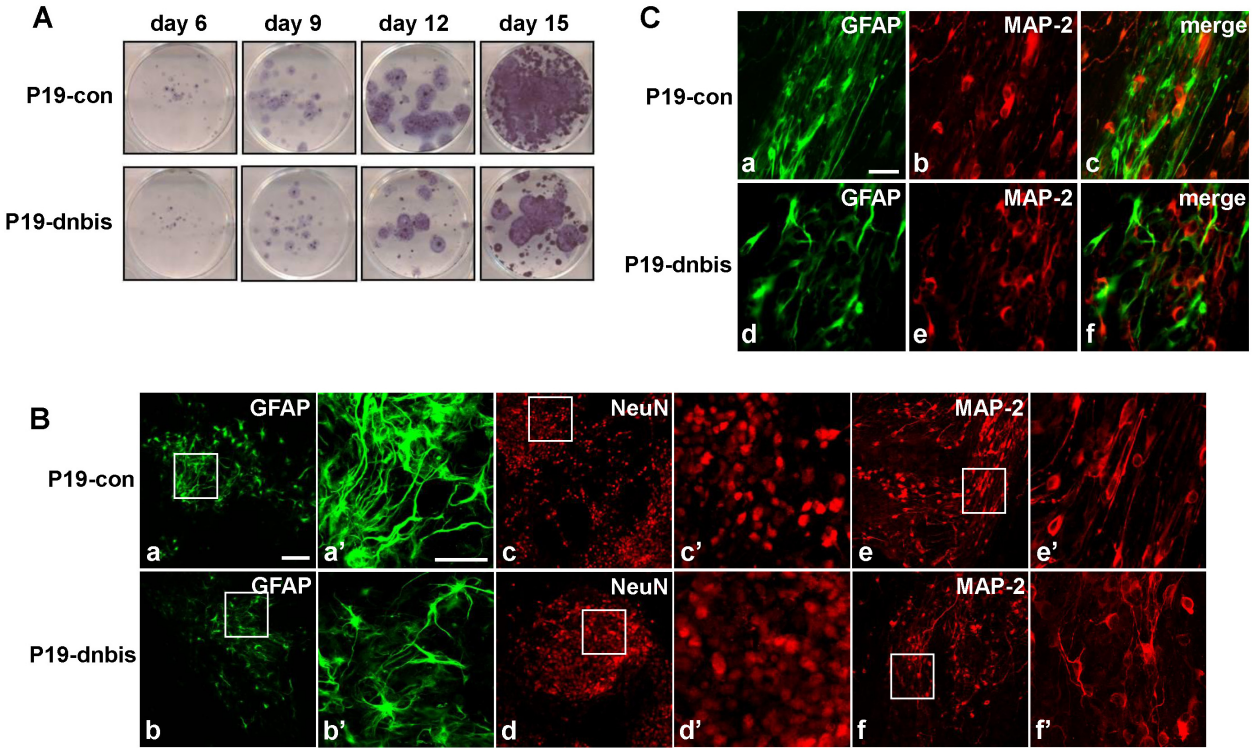

Fig. 3. The impairment of glial differentiation and neuronal distribution in Bis-knock down P19 cells after RA-induced differentiation. (A) The staining of viable cells after the induction of differentiation with RA in P19-con and P19-dnbis cells. After culturing cell aggregates in a Petri dish for 4 days, the cells were re-plated in 6 well tissue culture dishes and stained with MTT solution at the indicated days. (B) Confocal laser microscopic imaging of immunofluorescence for GFAP (a, b), NeuN (c, d) and MAP-2 (e, f) of P19-con (upper panels) and P19-dnbis (lower panels). a', b', c', d', e' and f' are higher magnifications of boxed areas of a, b, c, d, e and f, respectively. Scale bar, 50 μm (a−f) and 20 μm (a'−f'). (C) Confocal laser microscopic imaging of immunofluorescence for GFAP (a, d), MAP-2 (b, e) and overlay (c, f) of P19-con (upper panels) and P19-dnbis (lower panels) at day 12 after the induction of neuronal differentiation. Scale bar, 20 μm.

(Fig. 3A). The difference in the degree of expansion from embryonic bodies between P19-con and P19-dnbis became obvious at day 12, when GFAP induction was detected by RT-PCR and a Western assay (Fig. 2). These results suggest that the spreading or migration of differentiated neurons was impaired in P19-dnbis cells during RA-induced differentiation.

We then examined the expression patterns of glial and neuronal markers in P19-con and P19-dnbis cells. The population of GFAP-positive cells was lower in P19-dnbis cells, which is consistent with the quantitative analysis. In addition, a large proportion of GFAP-positive cells in the P19-con cells have an astral shape and diverse long processes while most of the processes of GFAP- positive cells in P19-dnbis show relatively short extensions (Fig. 3Ba, b). NeuN-labeled mature neurons in P19-con cells were widely dispersed whereas those in P19-dnbis cells were distributed in clusters (Fig. 3Bc, d). Furthermore, immunostaining with an MAP-2 antibody indicates that clusters of differentiating neurons with elongated bodies and long bipolar processes, which appear to be actively migrating, were occasionally found to be oriented in one direction whereas the MAP-2 positive cells in the P19-dnbis cells did not exhibit a unidirectional distribution (Fig. 3Be, f). The double labeling of MAP-2 and GFAP indicate that some MAP-2 positive cells have a close association with GFAP-positive cells, which was not observed in P19-dnbis cells (Fig. 3C). These results suggest that, neuronal migration is affected by a reduction in Bis expression.

DISCUSSION

Our results provide evidence to show that Bis is involved in gliogeneis or glial differentiation, which could affect the migration of neurons during in vitro neuronal differentiation. The relevance of Bis and glial differentiation was demonstrated by the following observations: First, Bis-expressing cells were partially co-localized with GFAP, but not with markers of neural differentiation. Second, the down-regulation of Bis resulted in a decrease in the induction of GFAP while NeuroD and Mash-1 was not affected by a decrease in Bis levels. Finally, time-course expressions for Bis indicated that the increase in Bis levels preceded the induction of GFAP, while NeuroD and Mash-1 expression was induced prior to the induction of Bis. Although a quantitative analysis indicated that the expression of neurogenic regulatory genes during RA-induced differentiation was not significantly influenced by the down-regulation of Bis, the suppression of Bis induction altered the allocation patterns of neuronal cells, as evidenced by an apparent attenuation or a delay in the spreading of differentiated neurons and the localization of NeuN-positive mature neurons in clusters in P19-dnbis cells.

The mechanism by which Bis modulates the distribution of neurons during RA-induced differentiation is currently unclear. Considering that Bis is not expressed in differentiated neurons but is, to some extent in some GFAP-positive glial cells, Bis might be involved in the activation of some function of glial cells, in addition to gliogenesis. It has previously been suggested that GFAP-positive cells are functionally involved in guiding migrating neurons during the neuronal differentiation of P19 cells (Liour and Yu, 2003). Large numbers of neuron-like cells that express neuronal marker proteins such as MAP-2 have been shown to migrate along GFAP positive radial glial-like cells from the edge to the outer stratum of an embryonic body (cell aggregates) (Santiago et al., 2005). These findings indicate that the migration of neuron-like cells requires a close association with radial glial-like cells (Liour and Yu, 2003; Santiago et al., 2005). Our results also show that the immunoreactivity of MAP-2 occasionally paralleled that of the GFAP after RA-induced differentiation of P19-con cells, which was not evident in bis-dnbis cells. GFAP-expressing cells in P19-dnbis also do not possess long processes, indicating that they lack function in guiding migrating neurons. We also performed double immunostaining with Bis and Nestin, another maker for radial glial cells, and found that they are not co-localized (data not shown). However, it has previously been proposed that radial glial-like cells revealed heterogenous phenotypes in P19 cells, as evidenced by the finding that GFAP-positive cells did not express Nestin (Liour and Yu, 2003). In support of this, Bis expressing cells in the RMS and the SVZ of the lateral ventricle are GFAP-positive astrocytes, but do not express Nestin (Park et al., 2003). Therefore, even though it is not certain if GFAP-positive cells are the only population of radial glial cells in our system, it can be postulated that Bis is implicated in the differentiation of some linage of radial glial cells.

The in vivo role of radial glial cells in neural development was already noted by showing that more than 80% of neuronal precursor cells migrate along the radial glial guide (Hatten, 1999). Based on the expression of Bis in the meshwork of GFAP-positive astrocytes that form glial tubes in SVZ and RMS, where neuronal differentiation and neurogenesis occur (Park et al., 2003; Choi et al., 2006), Bis might play a regulatory role of astrocytes in the neuronal differentiation and migration in SVZ and RMS in vivo. Very recently, Bis has been reported to be expressed in a subpopulation of radial glia cells in the developing brainstem and spinal cord, suggesting that Bis has a unique role in the association with the radial glial cells in the developing CNS (Choi et al., 2009, in press). Thus, the finding reported herein provide for the possibility that Bis is related to the function of radial glial cells, serving as scaffold for migrating neurons.

The SVZ neurogenesis in the adult has been shown to require a direct interaction with GFAP-positive astrocytes and SVZ precursors (Lim and Alvarez-Buylla, 1999). In addition, previous reports have shown that Bis regulates cellular motility and adhesion. In one study, the overexpression of Bis was reported to increase cellular motility and adhesion in human epithelial cancer cells and MEFs (Iwasaki et al., 2007). In another study, the overexpression of Bis was reported to decrease migration and adhesion in human breast cancer cells (Kassis et al., 2006). Therefore, it is possible that Bis expression in GFAP-positive glia cells might regulate the adhesion of neuronal cells with GFAP-positive astoryctes, enclosing migrating neurons from the surrounding parenchyma, thus guiding the migration of neuronal cells to their destination.

In conclusion, the suppression of Bis induction resulted in a decrease in gliogenesis and a decrease in the migration of differentiating neurons during RA-induced differentiation in P19 cells, suggesting that Bis is involved in the migration-inducing activity of glial cells.

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