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ORIGINAL PAPER

IGFBP-3 Inhibits the Proliferation of Neural Progenitor Cells

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Abstract Insulin like growth factor-1 (IGF-1) plays an important role in the proliferation and differentiation of neural progenitor cells. The effects of IGF-1 can be regulated by insulin like growth factor binding protein-3 (IGFBP-3) which can either inhibit or stimulate the proliferation of cells depending on the expression of proteases that can release IGF-1 from IGF1-IGFBP3 complex. Although IGF-1 is essential for the development of brain, both IGFBP-3 and IGF-1 are elevated in the brains of children younger than 6 months of age. Likewise, IGFBP-3 is also upregulated following cerebral ischemia and hypoxia. However, the role of IGFBP-3 in neurogenesis is not clear. Using an in vitro culture system of rat neural progenitor cells, we demonstrate that IGFBP-3 specifically regulates the IGF-1 mediated neural progenitor cell proliferation via down regulation of phopho-Akt, and cyclin D1. In addition, IGFBP-3 also decreased the content of nestin in the neural progenitor cells indicating its potential role in neurogenesis.

Keywords Neural stem cells · Cell cycle · Retinoblastoma · Neurogenesis

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Introduction

Insulin like growth factor-1 (IGF-1) is essential for the development of brain [1–3] playing an important role in the proliferation and differentiation [4–8] of cells. The effects of IGF-1 can be regulated by insulin like growth factor binding proteins (IGFBPs) which bind to IGF-1 with high affinity [2, 9] and regulate the bioavailability of free IGF-1 [10]. In this regard transgenic mice expressing IGFBP-1 have shown impaired brain development and reduced astrocyte response to injury [11]. IGFBP-3 is a hypoxia regulated factor which exerts anti-proliferative influence by inhibiting IGF-1 mediated mitogenic effects or through mechanisms that are independent of IGF-1 [12, 13]. Although, IGFBP-3 inhibits the effects of IGF-1, the inhibitory potential of IGFBP-3 depends on the concentration of IGFBP-3 and also proteases that degrade IGFBP-3 to release IGF-1. Therefore, the effect of IGFBP-3 on IGF-1 could be inhibitory or stimulatory depending on the expression of proteases which can degrade IGFBP-3 and release IGF-1 from IGF-IGFBP-3 complex [14]. The anti-proliferative effect of IGFBP-3 has been illustrated in epidermal keratinocytes in vivo [15]. The content of IGFBP-3 is low in the normal brain but its expression is upregulated following cerebral ischemia [16], thus IGFBP-3 may regulate the IGF-1 mediated proliferation in the post ischemic brain. Moreover, the expression of IGFBP-3 and IGF-1 are higher in children of less than 6 months age as compared to older children suggesting that they might participate in the myelination and synapse formation [3]. Hence, we investigated the effect of IGFBP-3 on the IGF-1 mediated proliferation of neural progenitor cells in vitro. The proliferation of cells was assessed by MTS assay and confirmed by total cell counting.

Experimental Procedure

Cell Culture

Adult rats (250–350 g) were obtained from Charles River Laboratories and were cared for in accordance with the NIH guide for the Care and Use of Laboratory Animals. Neurospheres were generated from the subventricular zone of the adult rat brain as described earlier [6]. All of the experimental procedures were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals. Briefly, rats were anaesthetized using isoflurane to minimize pain and the walls of the lateral ventricles were dissected, digested in an enzymatic cocktail containing papain (0.1%), dispase (0.1%), and DNase (0.01%). The dissociated cells were cultured in neurobasal medium containing B-27, glutamine (2 mM), FGF-2, (20 ng/ml), and heparin (2 μg/ml) until the progenitor cells formed neurospheres. The neurospheres were dissociated using accutase and cultured in neurobasal medium containing B27 (without insulin), FGF2 (20 ng/ml), IGF-1 (5 ng/ml) and/or IGFBP3 (5, 50, 500 ng/ml) and plated in 6 well plates (0.4×10^6 cells) for immunoblotting and cell counting, while 96 well plates (0.1×10^5 cells) were used for cell proliferation assay. All the experiments were performed between passages 3 and 20.

Electrophoresis and Immunoblotting

Neurospheres were homogenized in lysis buffer (20 mM Na_2HPO_4, pH 7.0, 50 mM NaF, 10 mM $Na_4P_2O_7$, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 2% Triton X-100, 1 mM Na_3VO_4 and 0.5% deoxycholate), centrifuged at 12,000*g* in an eppendorf centrifuge for 10 min to clarify the lysate. 30 μg of protein was separated by SDS–PAGE and transferred to PVDF membrane. The membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 followed by overnight incubation with primary antibodies for either phospho-Akt$^{(S473)}$, cyclin D1, phospho-Retinoblastoma (Ser$^{807/811}$), nestin or actin. After three washes peroxidase-coupled secondary antibody (anti-mouse IgG/anti-rabbit IgG) was added and incubated for 1 h. The membrane was washed and specific bands were visualized using super signal detection reagents (Pierce, Rockford, IL).

Cell Proliferation Assay and LDH Assay

Cell proliferation and LDH release were assessed from the same culture conditions. The LDH release was assessed by using Cyto Tox assay kit (Promega, Madison, WI). Cells (0.1×10^5) were cultured in 150 μl of neurobasal medium containing 2% B27 (without insulin), IGF-1 (5 ng/ml) and FGF2 (20 ng/ml) in a 96 well plate in the presence of varying concentrations of IGF-BP3 (0, 5, 50, 500 ng/ml) for 4 days. 50 μl of conditioned medium was used for LDH assay, while remaining 100 μl of medium containing cells was used to assess the cell proliferation. After 4 days in culture 50 μl of conditioned medium was transferred into another 96 well plate, mixed with 50 μl of LDH substrate solution and incubated for 30 min in dark at room temperature. The reaction was stopped by the addition of stopping solution (CytoTox assay kit) and the absorbance read at 490 nm. Same volume of medium was used for blank. The amount of LDH released is proportional to the intensity of the color. The remaining medium containing cells was incubated with 20 μl of cell proliferation assay reagent (CellTitre 96 AQ_{ueous}; Promega Madison, WI) at 37°C for 2 h and the optical density was measured at 490 nm. The same volume of medium without cells was used as blank.

Effect of IGFBP-3 on Cell Number

Neural progenitor cells (0.4×10^6) were cultured in the presence and absence of IGFBP-3 (500 ng/ml) for 4 days. At the end of experimental period neurospheres from control and IGFBP-3 treated cells were dissociated and counted using trypan blue staining. The total number of cells was assessed in both the conditions. Each experiment was performed 4 times.

Statistical Analysis

Statistical analysis was performed by ANOVA followed by Newman-Keuls multiple comparison test or students "*t*" test for two groups. Data is represented as mean ± S.D of 3–5 experiments and considered significant if $P < 0.05$.

Results

Incubation of neural progenitor cells with varying concentrations of IGF-1 increased the proliferation of cells as demonstrated by MTS assay (Fig. 1). IGFBP-3 significantly decreased the formation of neurospheres at 50 and 500 ng/ml as compared to control (Fig. 2a). The decrease in the neurosphere formation was consistent with a decrease in the metabolic activity of the cells as measured by MTS assay (Fig. 2b black bars). There was no cell death as measured by LDH assay and the amount of LDH released by the cells under various conditions was similar to control (Fig. 2b grey bars). Moreover, IGFBP-3 did not inhibit the insulin mediated proliferation of cells demonstrating its specific effect on IGF-1 (Fig. 3a). Consistent with these observations, IGFBP-3 decreased the total

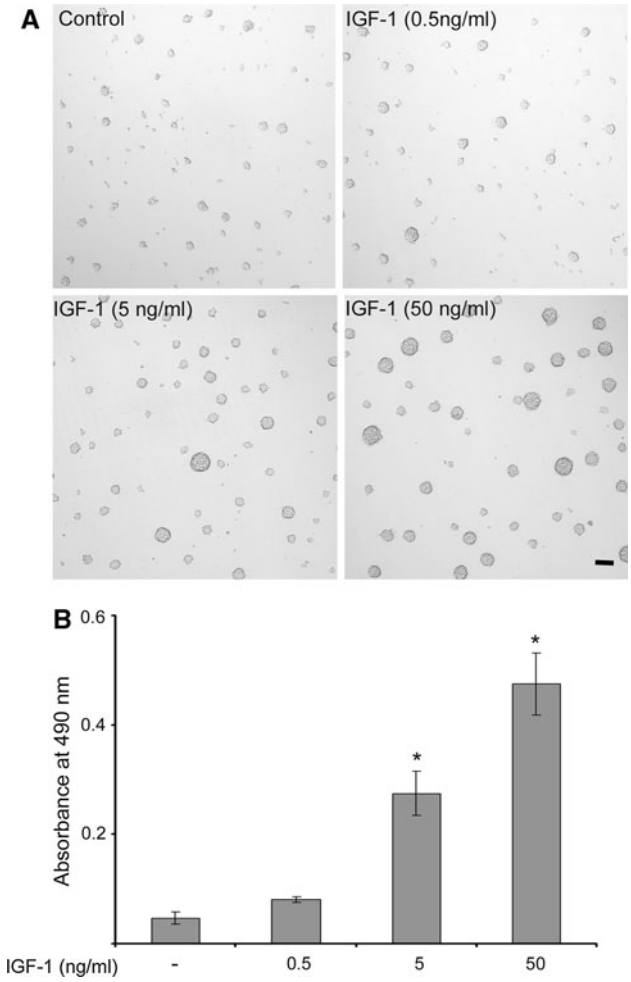

Fig. 1 IGF-1 (0.5–50 ng/ml) increases the proliferation of neural progenitor cells. Neural progenitor cells were cultured in neurobasal medium containing B27 (without insulin) and FGF2 under the following conditions (1) Control; (2) IGF-1 (0.5 ng/ml); (3) IGF-1 (5 ng/ml); (4) IGF-1 (50 ng/ml) for 4 days. **a** Neurosphere formation **b** Metabolic activity as measured by MTS assay. Note the increase in the proliferation of cells as a function of IGF-1 concentration. Data represented is mean ± SD of 3–6 individual experiments done in quadruplicate. *Scale bar*: 50 μm. *$P < 0.001$ as compared to control

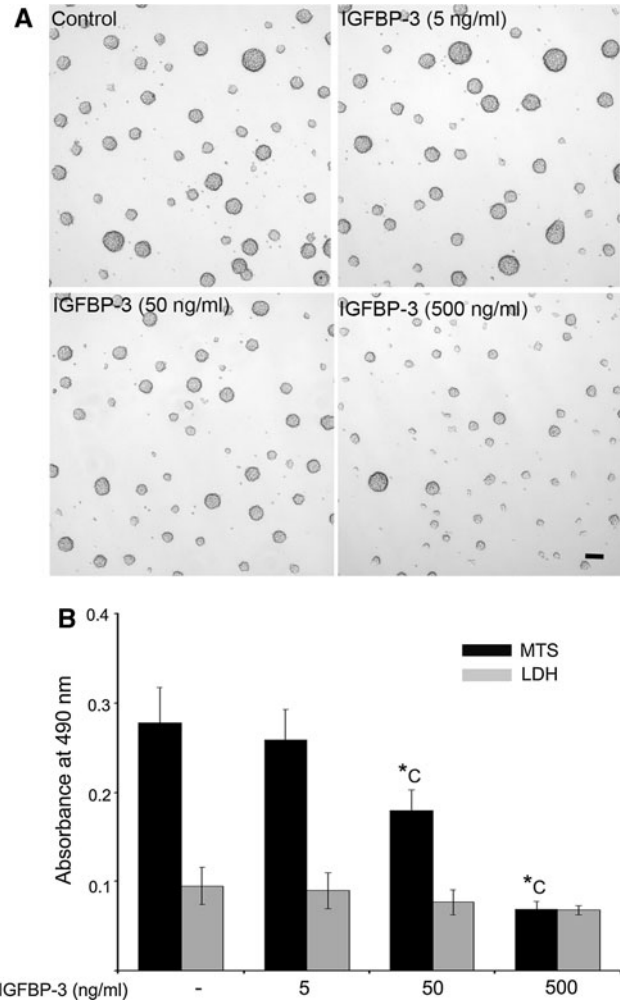

Fig. 2 Effect of IGFBP3 on the IGF-1 mediated proliferation of neural progenitor cells. **a** Neural progenitor cells were cultured as in Fig. 1 in a medium containing IGF-1 (5 ng/ml) and FGF2 (20 ng/ml) for 4 days in the presence of varying concentrations of insulin like growth factor binding protein 3 (0, 5, 50, 500 ng/ml). IGFBP3 decreased the formation of neurospheres at 50 and 500 ng/ml. *Scale bar*: 50 μm. **b** Metabolic activity and LDH release of neural progenitor cells cultured in (**a**). Note the decrease in the metabolic activity (*black bars*) but no change in the LDH (*gray bars*) release following IGFBP-3 (500 ng/ml) treatment. Data represents mean ± SD of 3–5 individual experiments performed in quadruplicate. *C $P < 0.001$ as compared to control

number of cells as compared to IGF-1 treated cells (Fig. 3b). In addition, incubation of neural progenitor cells with IGFBP-3 (500 ng/ml) decreased the phosphorylation of Akt, retinoblastoma and content of cyclin D1 & nestin (Fig. 4) with no significant change in actin.

Discussion

We examined the effect of IGFBP-3 on the IGF-1 mediated proliferation of neural progenitor cells. One difficulty in studying the effect of IGF-1 on the proliferation of neural progenitor cells is the presence of insulin contained in the B27 supplement. Insulin can stimulate IGF-1 receptors and thus can interfere with the specific IGF-1 signaling. Hence we examined the effects of IGF-1 on the proliferation of neural progenitor cells using neurobasal medium containing B27 (without insulin) and FGF2. Our results show an increase in the proliferation of cells following incubation with IGF-1 in a concentration dependent manner (Fig. 1). These results are consistent with the metabolic activity of the cells and establish the effect of IGF-1 on the proliferation of cells.

It has been shown that IGFBP-3 may regulate the bioavailability of free IGF-1 and was implicated in the

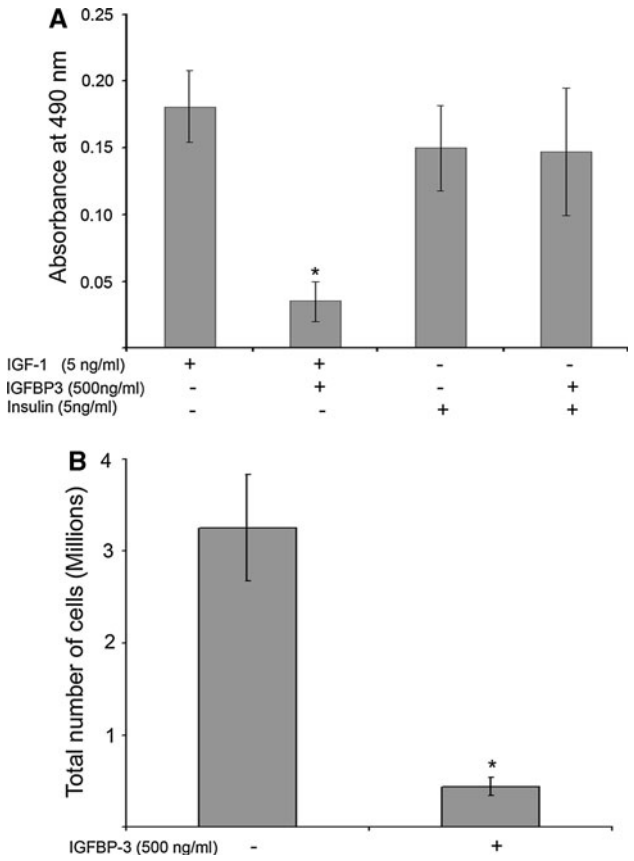

Fig. 3 **a** IGF-1 dependent effect of IGFBP-3 on the proliferation of cells. Metabolic activity (MTS assay) of neural progenitor cells cultured in neurobasal medium containing B27 (without insulin), FGF2 and IGF-1 (5 ng/ml) or Insulin (5 ng/ml) in the presence and absence of IGFBP-3 (500 ng/ml) for 4 days. Data represents mean ± SD of 5–6 individual experiments performed in quadruplicate. *C $P < 0.001$ as compared to control. **b** IGFBP-3 decreases the proliferation of neural progenitor cells. Neural progenitor cells (0.4×10^6) were cultured in neurobasal medium containing B27 (without insulin), FGF2 (20 ng/ml), IGF-I (5 ng/ml) in the presence and absence of IGFBP-3 (500 ng/ml) for 4 days. At the end of experimental period neurospheres were dissociated and the total number of cells was counted using trypan blue staining. IGFBP-3 decreased the total number of cells as compared to control. Data represented is mean ± SD of 4 individual experiments. *$P < 0.001$ as compared to control

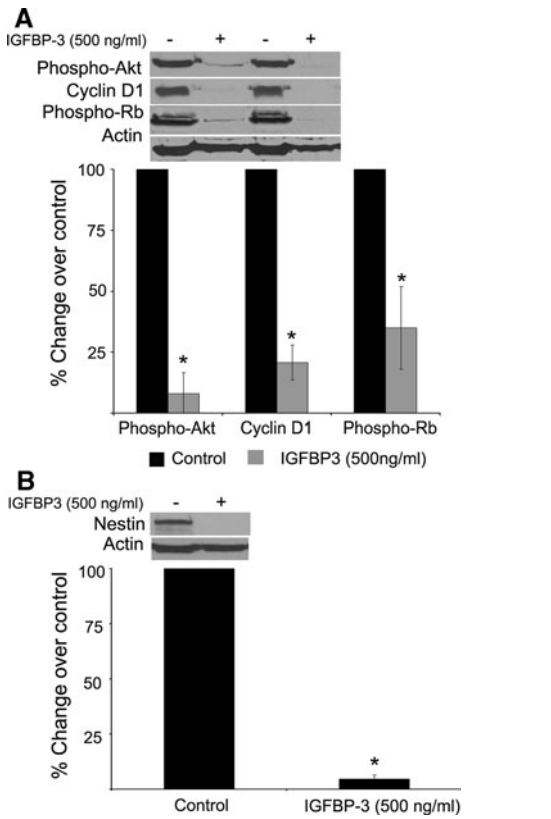

Fig. 4 Effect of IGFBP3 on the phospho-Akt, phospho-Rb, cyclin D1 and nestin content. Neural progenitor cells were cultured as in Fig. 3b in the presence and absence of IGFBP-3 (500 ng/ml) for 4 days. The neurospheres were used for immunoblotting. **a** IGFBP-3 decreased the phosphorylation of Akt, Rb & cyclin D1 content. $n = 2$ out of 3 experiments. **b** IGFBP-3 decreased the content of nestin. All the *bands* are normalized to actin and expressed as % change over control. *$P < 0.01$ as compared to control. Each experiment was repeated 3 times

inhibition of IGF-1 mediated proliferation of cancer cells [17]. However its role in the proliferation of neural progenitor cells is not clear**.** Generally the sequestration of IGF-1 by IGFBP-3 may result in either a decrease in the bioavailability of IGF-1 or an enhanced stability of IGF-1 [18] depending on the content of IGFBP-3 [19] or the activity of IGFBP-3 proteases [20] which degrade IGFBP-3 and release IGF-1. IGFBP-3 decreased the formation of neurospheres at 50 and 500 ng/ml (Fig. 2a). The decrease in the formation of neurospheres was consistent with a reduced metabolic activity of the cells (Fig. 2b). It is interesting to note that higher concentrations of IGFBP-3 (10 & 100 fold) are required to inhibit the IGF-1 mediated proliferation of neural progenitor cells. A recent study has shown that equimolar ratio of IGFBP-3 (10 nM) inhibited the IGF-1 (10 nM) mediated proliferation of placental cells [12]. Several studies have demonstrated that IGF-1 is an anti-apoptotic factor which enhances the cell survival [6, 21] hence IGFBP-3 may decrease the survival of the cells by inhibiting the effects of IGF-1. Therefore, we assessed the cell death by measuring the lactate dehydrogenase (LDH) released by the cells into the medium. Our results show that the amount of LDH released by IGFBP-3 treated cells was similar to control cells (Fig. 2b) indicating that IGFBP-3 did not induce cell death. These results suggest that either IGF-1 is not completely sequestered by IGFBP-3 or is released by the slow degradation of IGFBP-3 by IGFBP-3 proteases. It is also possible that some other component in the culture medium may be responsible for preventing cell death in the absence or very low content of IGF-1. A previous study has shown that IGFBP-3 promotes

cytoprotection following vascular injury [22]. Moreover, IGFBP-3 did not inhibit the proliferation of cells in the presence of insulin illustrating its specific effect on IGF-1 (Fig. 3a). Further studies on total cell number established an inhibitory role for IGFBP-3 in the IGF-1 mediated proliferation of neural progenitor cells in vitro (Fig. 3b).

IGF-1 stimulates the phosphorylation of Akt, but not ERK and regulates the expression of cyclin D1 [6, 23, 24], therefore we studied the effect of IGFBP-3 on phospho-Akt content. Likewise, Cyclin D1 activates CDK4/6 to phosphorylate retinoblastoma protein to increase the proliferation of cells. Hence, D type cyclins are critical for the entry of cells into the cell cycle, however GSK-3β mediated phosphorylation of cyclin-D1 at Thr^{286} induces the proteosomal degradation of cyclin D1 [25]. IGF-1 blocks the phosphorylation of cyclin-D1 by inhibiting GSK-3β activity to enhance the stability of cyclin D1 content [23]. Consistent with these observations our results show that IGFBP-3 decreased the phosphorylation of Akt and down regulated the cyclin D1 content (Fig. 4a). The decline in the cyclin D1 content decreased the phosphorylation of retinoblastoma to inhibit cell division. A recent study has shown that over-expression of cyclin D1/CDK4 in neural progenitor cells can shorten the G1 phase to increase the proliferation, while knockdown of cyclin D1/CDK4 prolonged the G1 phase and induced neurogenesis [26]. In line with the above studies our results show that IGFBP-3 decreased the content of nestin (Fig. 4b), a marker of stem/progenitor cells, suggesting the differentiation of cells. Hence it is reasonable to speculate that IGFBP-3 may facilitate the differentiation (neurogenesis) by inhibiting the proliferation of neural progenitor cells. Further studies are required to address these questions.

In summary we show that IGFBP-3 inhibits the IGF-1 mediated proliferation of neural progenitor cells by curtailing the Akt signaling and down regulating the cyclin D1 content which results in decreased phosphorylation of retinoblastoma and subsequent cell cycle arrest.

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