Original Article

The Role of Wnt Signaling Pathway on the Expression of TGFβ 1 and TGFβ 2 in Cultured Rat Cortical Astrocytes

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Abstract

Introduction: Astrocytes, the most abundant glia in the central nervous system, modulate neuronal survival and function. Astrocytic functions are mediated by synthesis and secretion of wide ranges of polypeptides through mechanism (s) poorly understood. Among these, TGFβs are synthesized and released by the astrocytes. In this study, the involvement of Wnt signaling pathway on the synthesis of TGFβs by the astrocyte was investigated.
Materials and Methods: Cultured rat astrocytes were therefore treated either with Wnt3a (20 ng/ml) alone for 24 hours or in combination with sFRP-1 (400 ng/ml) for a further 24 hours. Cells were then harvested and examined for the expression of TGFβs and the Wnt target gene, cyclin D1.
Results: In this study, we were able to show that 1) treatment Wnt3a alone for 24 hours induced the expressions of TGFβs and cyclin D1; 2) The effect of Wnt was inhibited by pre-treatment with sFRP-1, that is, sFRP-1 pre-treatment significantly blocked the Wnt-induced expressions of TGFβs and cyclin D1.
Conclusion: This study therefore provides the first evidence for the involvement of Wnt signaling pathway in the synthesis of TGFβ proteins by cortical rat astrocytes.
Keywords: Astrocytes, Wnt3a Protein, Transforming Growth Factor beta (TGF beta), Secreted frizzled related protein-1 (sFRP-1), Cyclin D1

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Introduction

Glial cells comprised of the astrocytes, oligodendrocytes and microglial cells are the largest cell populations in the central nervous system (CNS). Astrocytes support neurons by regulating their activity and synaptic transmission [1-2]. These functions are mediated by release of various amino acids and polypeptides. Among these peptides, the family of transforming growth factor beta (TGFβ) are known to be produced and released by astrocytes [3], mediating astrocyte-induced neuroprotection. TGFβ is a member of the TGFβ super family consisting of 3 isoforms in mammals (TGFβ 1, 2, and 3), each encoded by different genes. Astrocytic secretion of TGFβ is known to be regulated by several important signaling pathways including phosphatidylinositol 3-kinase (PI3K/Akt) [4]. Wnt proteins (Wnt1 and Wnt 3) signal through a receptor complex composed of members of the Frizzled (Fz) and low-density lipoprotein receptor-related protein (LRP) families, and activate a number of intracellular signaling pathways including the β-catenin/TCF pathway (known as the canonical Wnt pathway) [5-6]. The present study aimed at if the synthesis of TGFβs 1 and 2 and cyclin D1 as a target gene in the Wnt pathway are affected by Wnt3a or its inhibitor, secreted frizzled related protein (sFRP-1).

Materials and Methods

Astrocyte culture and treatments

All study procedures were approved by the the Ministry of health animal care Ethics Committee, Tehran, Iran. Brains from 1-4 days old Wistar rat pups (Pasteur institute of Iran) were used for the astrocyte culture. Astrocytes were separated from neural and non-neural cells according to McCarthy and colleagues, and prepared for the treatments [7]. Briefly, following removal of meninges, cortices were dissected, washed in DMEM media, cut into small pieces and homogenized in complete DMEM by triturating. Complete culture medium contained DMEM (Gibco, Germany) supplemented with 20% fetal bovine serum (FBS; SPL life sciences, Korea) and antibiotics (penicillin and streptomycin; Gibco, Germany). The brain homogenate was then placed in a humidified cell culture incubator under an atmosphere of 5% CO2 at 37ºC. After a couple of days, flasks became confluent (filled with cells by 70%), the cells were trypsinized and transferred to new flasks 25 cm^2 flasks (Nunc, Germany) at $5x10^5$ cells containing medium with 10% FBS. Since astrocytes are the quickest among the glials and also compared with neurons to attach, they were isolated from the other glials by changing the culture medium after a few hours. This led to the removal free floating neurons, oligodendrocytes and microglial cells. The purity of the astrocytes was assessed by immunostaining with glial fibrillary acidic protein (astrocyte specific marker; GFAP; Roche, Germany) that confirmed a purity of 95% (Fig. 1). After 10 days, a point at which cultures became confluent, cells were cultured in new flasks, treated with Wnt3a (20 ng/ml; R& D systems, Canada) and sFRP-1 (400 ng/ml; Peprotech, Canada). Wnt was applied either alone for 24 hours or in sFRP-1 pre-treated astrocytes. For all experiments, the viability was assessed before and after the treatments using trypan blue and typically over 90% of the cells excluded the dye. The used doses of sFRP-1 and Wnt did not induce toxicity and did not change cell viability.

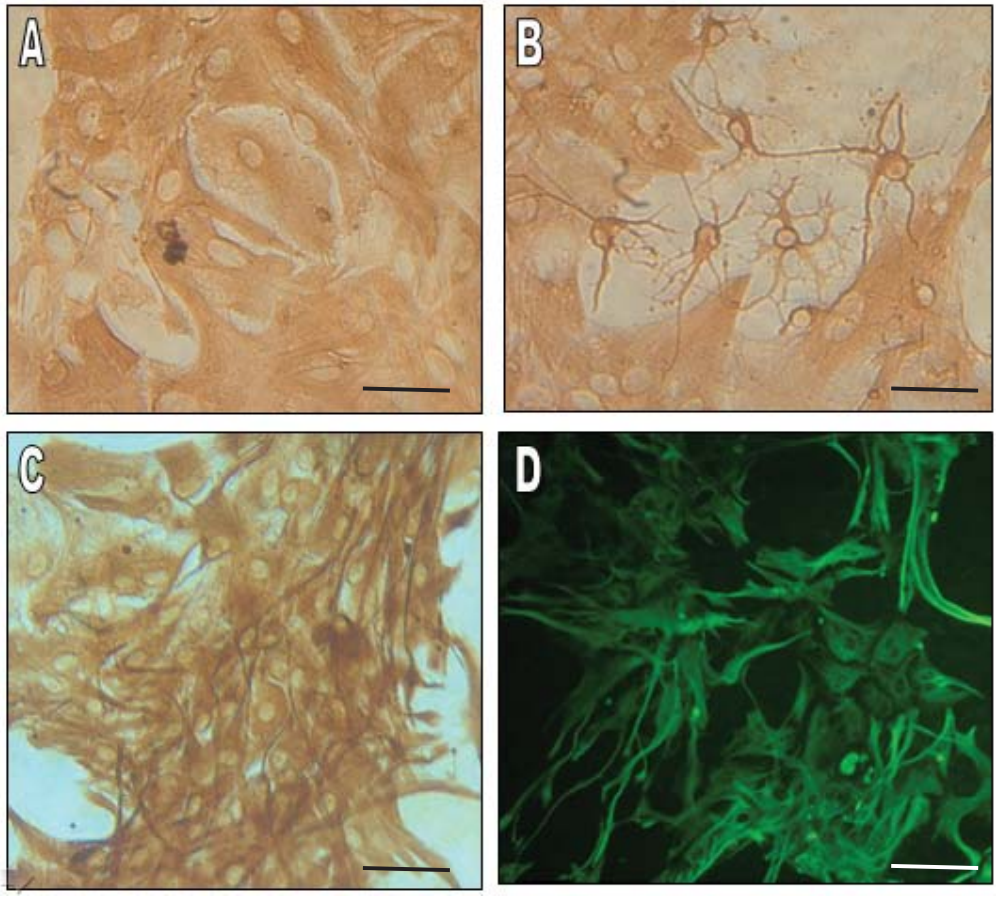

Figure 1. GFAP immunostaining in cultured adult rat astrocytes. (A-C) Detection of GFP immunoreactivity using DAB chromogen. D) using FITC fluorochrome, Scale bar: 250 μm.

Immunocytochemistry

20,000 cells of a second passage were plated per well/12-well plate. For GFAP immunostaining, cells were fixed by cold methanol and permeabilized using Triton-X100 (0.25%; Merck, Germany). They were then blocked by 3% BSA (Merck, Germany), followed by overnight incubation with anti-GFAP (1:200; Sigma, Germany), 2 hours in biotinylated secondary antibody (Eskanteb, DAKO, Netherlands) and 1 hour in FITC-streptavidin (Eskanteb, DAKO, Netherlands) for detection under the fluorescence microscope.

Real-time PCR

Total RNAs were extracted by using easy blue RNA extraction kit (iNtRON, Korea). cDNAs were synthesized by Fermentas kit (Nedayefan, Iran). Using Roche Light Cycler, real time PCR was performed for quantification of the levels of β-actin, TGFβs and cyclin D_1 mRNAs. The sequences of the primers (Roobinteb gostar, metabion, Germany) for beta actin were:
5`AAGGCCAACCGTGAAAAGAT 3` and 5'ACCAGAGGCATACAGGGACA3`, for TGFβ1 F: 5`CCTGGAAAGGGCTCAACAC 3`
R: 5`CAGTTCTTCTCTGTGGAGCTGA 3`, for TGF β2 F: 5`AGTGGGCAGCTTTTGCTC 3`

R: 5`GTAGAAAGTGGGCGGGATG 3` and those for cyclin D_1 were forward:
5`GCCACCTGGATGCTAGAGG3`and reverse:

5`CAGGCGGCTCTTCTTCAG3`. Real time PCR for TGFβs, beta actin and cyclin D_1 was performed according to the following program: (95 ºC: 5 min, cycles of 95 ºC: 10 sec, 60 ºC: 30 sec). Product specificity was confirmed by melting curve analysis and visualization of a single band of the appropriate product size on a 2% agarose gel. (Fanavariteb, Invitrogen, Germany). Expression levels were quantified by a standard curve using cDNA dilutions, and gene levels were normalized to the house keeping gene beta actin and compared with those in the controls. According to the method of Pfaffl and colleagues data were expressed as the fold changes compared with vehicle-treated cultures, using three per group and triplicates for verification of results [8].

Statistical analyses

Each experiment was performed in triplicates and the data obtained were analysed by SPSS Statistics software (version 19). One way ANOVA and the post-hoc test LSDs were used to determine the significance of variations. The P values < 0.05 considered as significant.

Results

Wnt 3a induces the expression of TGFβs and cyclin D1 in the cortical astrocytes

Real time PCR analysis of the treated astrocytes showed that in cells treated with Wnt 3a (20 ng/ml) for 24 hours the levels of $TGF\beta1^{mRNA}$, $TGF\beta2^{mRNA}$ and cyclin $D1^{mRNA}$ were increased by 0.68±0.05, 0.95±0.07 and 0.67±0.02 times respectively (Fig. 2), indicating that Wnt ligand activates the Wnt signaling pathway and induces the expression of TGFβs in the astrocytes.

The expression of TGFβs and cyclin D1 is suppressed by Wnt antagonist, sFRP1.

Treatment of the astrocytes with specific antagonist for canonical Wnt pathway, sFRP-1 for 24 hours followed by Wnt-3a for another 24 hours, the levels of $TGF\beta1^{mRNA}$, $TGF\beta2^{mRNA}$ and cyclin $D1^{mRNA}$ reduced significantly by 0.26±0.03, 0.47±0.04 and 0.24±0.08 times respectively (Fig. 2), indicating that the synthesis of TGFβs is specifically inhibited by Wnt antagonist in the astrocytes.

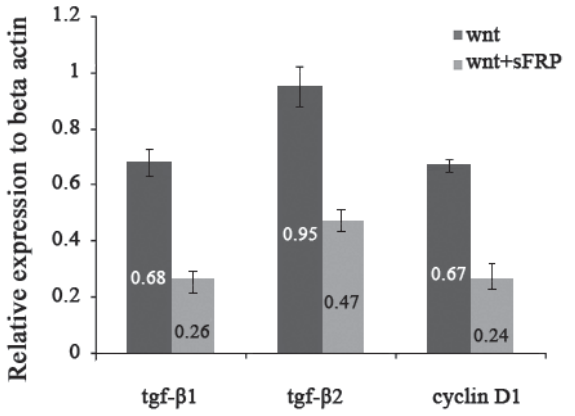

Figure 2. Real time PCR analysis of $TGF\beta1^{mRNA}$, $TGF\beta2^{mRNA}$ and cyclin $D1^{mRNA}$ in the cortical astrocytes treated with sFRP-1 for 24 hours followed by Wnt-3a for another 24 hours. While Wnt 3a induced the expression of the candidate genes significantly, co-treatment with sFRP-1 decreased their expression.

Discussion

Astrocytes produce large ranges of soluble and membrane associated signals which affect their neighbors and influence the development of the central nervous system [9]. Among the soluble factors released by the astrocytes, the family of TGFβ [10], are known to regulate the astrocyte physiology. So far, very few data is available on the mechanism (s) involved in the synthesis of neuroprotective factors released by the astrocytes. Considering that Wnt and its receptor/Co-receptor are expressed in the astrocytes [11-12], one proper candidate could be Wnt signaling pathway. To investigate this, we have applied Wnt ligand (Wnt3a) and a Wnt antagonist (sFRP-1) in cultured astrocytes and measured the

expression levels of TGF betas. Wnt antagonists could block the Wnt pathway upstreamly either by binding directly to the Wnt ligand or indirectly to its receptor/co receptor (Frizzled/LRP5/6). There are also downstream blockers of Wnt pathway, e.g. through blocking GSK-3 (glycogen synthase kinase) a rate limiting enzyme. We have previously shown that treatment of cultured astrocytes with a specific inhibitor of GSK-3 (BIO), enhances the expression of TGF betas [13]. However, since GSK-3 is a common key element of other signaling pathways such as PI3K/Akt pathway [14], in this study we sought to activate/blockade the Wnt pathway at upstream to clarify the specificity of the Wnt involvement. Indeed, the synthesis of TGF betas in the astrocyte treated with Wnt ligand (Wnt3a) was increased, whereas in those combined with Wnt antagonist (sFRP-1) was decreased. Also, we further examined the downstream key element of the Wnt pathway, cyclin D1 and showed that it was increased by Wnt3a and decreased following the sFRP-1 pre-treatment. Altogether, our results indicate for the first time that upstream and downstream activations of the Wnt pathway in the astrocytes both lead into one direction, that is, the induction of TGF beta synthesis. L'Episcopo and colleagues have also provided an indirect evidence for the involvement of Wnt in neuroprotection by astrocytes [15]. They have suggested the existence of an autoprotective loop between the astrocyte-dopaminergic neurons. Co-culturing dopaminergic neurons with midbrain astrocytes, phenocopies Wnt1 and induces neuroprotective effects, whereas RNA interference- mediated knockdown of Wnt1 in midbrain astrocytes markedly reduces astrocyte-induced TH+ neuroprotection [15]. Lie and colleagues have also shown that factors derived from the hippocampal astrocytes activate Wnt/beta catenin pathway and induce the differentiation of hippocampal neural stem cells [11]. Kornyei and colleagues have shown that interaction of Wnt with other secreted factors from glia affects neural cell fate [16]. Altogether, there is a possibility of autocrine activity of the Wnt3a, secreted by the astrocytes, to protect neurons. The expression of frizzled receptors on the astrocytes would also mediate this autocrine activity [12]. Wnt could also affect on development of astrocyte progenitors. Liu & Nathans have shown that in Fz5-/- mutant mice, there is an excess of astrocyte precursors and mature astrocytes, indicating that Wnt inhibits the differentiation of the astrocytes [17]. Feigenson and colleagues have also shown a Wnt inhibitory effect on oligodendrocyte differentiation [18]. Although one could never rule out the interaction of Wnt with other signaling pathways such as PI3K/Akt pathway which have common downstream key element (s) such as GSK-3, resulting in similar effects. As pointed out by Dhandapani and colleagues, blocking PI3K/Akt pathway in the astrocyte, inhibits the synthesis and secretion of TGFβs by beta estradiol [4]. In conclusion, Wnt signaling pathway is an effective route for neuroprotective actions of the astrocytes which may interact with other signaling pathways such as PI3K/Akt pathway to regulate the physiology of neuron-glia. Future studies would be required to elucidate the redundancy or complementary actions of these pathways.

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