ORIGINAL ARTICLE

# Effect of TGFβ1 and nAG on Astrocyte Cultures: A Study of Astrocyte Proliferation and the Expression of GFAP, CSPG4, S100B and IL-6

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## ABSTRACT

**Objective:** To determine the effects of TGFβ1 and an anti-scar protein nAG on normal (un-injured) 3-D astrocyte cultures with special emphasis on astrocyte proliferation, and the expression of GFAP, CSPG4, S100B, and IL-6.
**Study Design:** An experimental study.
**Place and Duration of Study:** King Saud University, Riyadh, Saudi Arabia, from March to September 2017.
**Methodology:** 3-D astrocyte gels were treated differently to create one control group (control, untreated astrocytes) and five experimental groups: nAg-only (treated astrocytes with 1 nM nAG recombinant protein), TGFβ1-only (treated astrocytes with 10 ng/ml TGFβ1), TGFβ1+low nAg concentration, TGFβ1+intermediate nAg concentration, and TGFβ1+high nAg concentration. Astrocyte proliferation, and the expression of GFAP, CSPG4, S100B, and IL-6 were studied and compared in these experimental groups.
**Results:** There were major differences in the responses of normal astrocytes *in vitro versus* the responses of astrocytes *in vivo* in the setting of injury or disease. The effects of TGFβ1 were dominant over the effects of nAG with regard to changes in CSPG4 and S100B by Real-Time PCR. There was a synergistic inhibitory effect of TGFβ1 and nAG on the expression of CSPG4 by Western blot.
**Conclusion:** The different responses of normal astrocytes *in vitro versus* astrocytes *in vivo* in the setting of injury or disease. The response at the gene level might not coincide with the response at the protein level.

**Key Words:** *TGFβ1, nAG, Astrocyte culture, GFAP, CSPG4, S100B, IL-6.*

## INTRODUCTION

Injured axons of the human central nervous system are unable to regenerate. Although there are many factors which contribute to this regeneration failure, it is now well established that glial scar is the main factor.[1] Glial scars are produced by astrocytes. Following trauma, astrocytes show hypertrophy of their intermediate filament proteins, mainly the GFAP (Glial Fibrillary Acidic Protein), and also excrete excessive amounts of proteoglycans. The most abundant proteoglycans produced by these reactive astrocytes are the chondroitin sulfate proteoglycans (CSPG).[2]

Transforming growth factor beta 1 (TGFβ1) is thought to be an important trigger for reactive gliosis. However, blocking TGFβ1 (using antibodies) does not prevent glial scar formation and injured axons are still unable to regenerate.[1] Furthermore, other authors have shown that the effect of TGFβ1 on astrocytes is not consistent and is affected by the strain rate produced by the mechanical insult.[3]

The newt-Anterior Gradient (nAG) protein is the main protein responsible for the regeneration of amputated limbs in salamanders. The protein is expressed by the Schwann cells of regenerating axons at the amputation stump. Al-Qattan *et al.* designed a nAG plasmid and recombinant protein suitable for use in higher vertebrates and demonstrated the strong anti-fibrotic properties of nAG in human and rabbit fibroblasts.[4,5] More recently, the authors demonstrated that the local injection of nAG protein into a crushed area of the spinal cord in rats will also reduce gliosis.[6] However, the molecular basis of this reduced gliosis has not been studied.

The objective of this study was to determine the effects of TGFβ1 and nAG on astrocyte cultures with special emphasis on astrocyte proliferation, and the expression of GFAP, CSPG4, S100B, and Interleukin 6 (IL-6).

## METHODOLOGY

Approval for this *in vitro*, experimental study was obtained from Riyadh Care-National Hospital. It was conducted at King Saud University, Riyadh, Saudi Arabia, from March to September 2017. Normal human astrocytes (NHA) (Lonza, Allendal, USA) were cultured and maintained in complete medium which include

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*Received: January 22, 2018; Accepted: July 18, 2018*

Astrocyte cell basal medium ABM (Lonza) and AGM Single Quots (Lonza) as growth supplements (rhEGF, insulin, ascorbic acid, gentamycin, L-glutamine and FBS). The cells were seeded in poly-D-lysine 75 $cm^2$ coated flasks (Corning, New York, USA). The cells were subcultured when 70-80% confluent and contain many mitotic figures through the flask.

Cells were seeded at a density of 2-million cells/ml gel, as previously described.[7] The gels were performed by mixing 80% type-I rat tail collagen 2 mg/ml (Gibco, Carlspad, California, USA) with 10% 10x minimum essential medium (MEM) (Gibco), neutralised by sodium hydroxide till color change to faint pink. The collagen-MEM mixture was gently mixed with 10% cell suspension in ABM medium and transferred to 24-well plates (750 ul/well) and placed at 37°C for 5 minutes. The solidified gels were covered with 2 ml ABM complete medium and placed in 5% $CO_2$ incubator at 37°C.

The 3-D astrocyte gels were treated differently to create one control group (control, untreated astrocytes) and five experimental groups: nAg-only (treated astrocytes with 1 nM nAG recombinant protein; Genscript, Piscatway, NJ, USA), TGFβ1-only (treated astrocytes with 10 ng/ml TGFβ1; PeproTech, Rocky Hill, NJ, USA), TGFβ1+low nAg concentration (treated astrocytes with 10 ng/ml TGFβ1 and 1 pM of nAG), TGFβ1+intermediate nAg concentration (treated astrocytes with 10 ng/ml TGFβ1 and 1 nM of nAG), and TGFβ1+high nAg concentration (treated astrocytes with 10 ng/ml TGFβ1 and 100 nM of nAG).

Treatments were done every other day taking into account the volume of the gel in calculating the treatments concentrations. Treatments were performed for 15 days and all experiments were repeated three times. Means and standard deviations were calculated for statistical analysis.

Proliferation activity of normal human astrocytes (NHA) were assessed by incorporation of Bromodeoxyuridine (BrdU, Roche, Mannheim, Germany). Astrocytes in 100 ul gels were seeded in 96 well plate. Cells were labeled using 10 μM BrdU per well and incubated for two hours at 37°C in a humidified atmosphere. After fixation, the cells were incubated with the anti-BrdU-POD antibody for 90 minutes at room temperature. After removal of the antibody conjugate, cells were washed and the substrate solution was added. The reaction product was quantified by measuring the absorbance using Microplate Reader Synergy 2 (BioTek, Winooski, Vermont, USA) at 370 nm.

Total RNA was extracted from normal human astrocytes (NHA) in collagen gels using Trizol (Ambion, Foster City, USA). One ml Triazol was added to each well after medium removal and PBS wash. The gels were homogenised in Triazol by a needle on a syringe until no clumps of collagen gel remained. RNA extraction was then performed according to the manufacturer's instructions. Equivalent amounts of total RNA (10 ng) were reverse-transcribed and target genes were amplified in one-step reaction using Quantitect SYBR Green kit (Qiagen, Dusseldorf, Germany) and Quantitect primer assay (Qiagen) pre-designed primers for GFAP, CSPG4, and S100B. The reactions were set up as per manufacturer's directions. Thermal cycling conditions were as follows: an initial reverse transcription step for 30 min at 50°C, incubation for 15 min at 95°C to activate hot star Taq DNA polymerase; and then 40 cycles as follows: denaturation for 15 sec at 94°C, annealing for 30 sec at 55°C and extension for 30 sec at 72°C. Target genes were normalised to the GAPDH gene (the gene encoding Glyceraldehyde 3-phosphate dehydrogenase protein; which is unaffected by teatments) and relative concentration were determined using ct method in Rotor-gene Q 5 plex software (Qiagen).

Total proteins were extracted from NHA in gels by RIPA cocktail (Santa Cruz, Texas, USA). The medium was removed, gels were washed with PBS, transferred into 2 ml low binding protein tube and covered with 200 ul RIPA. After homogenisation with needle, cell suspensions were centrifuged at 14,000 rpm for 15 minutes at 4°C. Protein concentration was measured by Bradford reagent (Sigma, Louis, USA).

Equal amounts of proteins (100 ug) were separated on SDS polyacrylamide gel then proteins were transferred into nitrocellulose membranes (Invitrogen, Carlsbad, California, USA) using Mini Protean Trans blot (Bio-Rad, Hercules, California, USA) at 100V for one and a half hour. After the transfer, membranes were blocked by 5 % blocking buffer Blotto (Santa Cruz) dissolved in TBST for one hour at room temperature. The membranes were then incubated with primary antibodies dissolved in blocking buffer as follows: anti-NG2 (abcam, Eugene, USA) (1:200) for CSPG4 detection, anti S100 (abcam) (1: 200) for S100 detection, and anti GAPDH (Santa Cruz) (1:1000) for GAPDH detection. After three washes, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (Santa Cruz) dissolved in blocking buffer (1:10000) for one hour at room temperature. After washing, the immunoblots were visualized by ECL immunocruz kit (Santa Cruz) and the bands densities were quantified using Quantity One software (Bio-Rad) Results were expressed as ratio of band density to GAPDH.

A quantitative sandwich ELISA for IL-6 (abcam) was performed according to the manufacturer's instructions. Briefly, a standard curve for human IL-6 was prepared (range 0-50 pg/ml). Hundred μl of each standard, sample (cell culture supernatant) and blank control were added to wells pre-coated with human-specific IL-6 capture antibody. After incubation for three hours at

room temperature with 50 ul biotinylated anti-IL-6, the wells were washed then incubated with 100 ul of streptavidin-HRP for 30 minutes at room temperature. Following further washing, a 100 ul of chromogen TMB substrate solution was added to the wells and incubated for 15 minutes at room temperature in the dark. The enzyme reaction was stopped using 100 ul of stop reagent and the absorbance of each well was read at 450 nm, with reference reading at 620 nm using micro plate reader Synergy 2 (BioTek, Winooski, Vermont, USA).

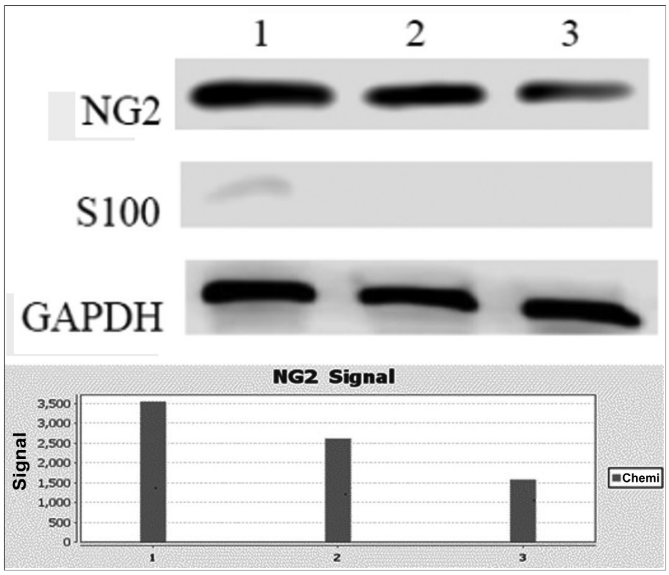


**Figure 1:** Western blot was done for cell lysates of control astrocytes (lane 1), TGFβ1 treated astrocytes (lane 2) and TGFβ1+ nAG 1nM treated astrocytes (lane 3) for detection of NG2, S100 and GAPDH as loading control. There was decrease in NG2 and inhibition in S100 protein expression in TGFβ1 and TGFβ1+ nAG treated cells. The least NG2 protein expression was in astrocytes treated with TGFβ1+ nAG. Note that NG2 is the measure of CSPG4.

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 22 software (SPSS Inc., Chicago, IL, USA). Calculated means and standard deviations for numerical (measurable) variables were done. One-way analysis of variance (ANOVA) was utilised first to compare results of experiments of different groups; and then by using post-Hoc tests for multiple comparisons to determine significant differences where p-value less than 0.05 was considered significant.

## RESULTS

There were no significant differences in proliferation between astrocytes with different treatments compared to control untreated astrocytes (p =0.469, Table I). For GFAP, there was a significant decrease in mRNA expression (compared to control) in astrocytes treated with TGFβ1 only (p=0.001), astrocytes treated with TGFβ1+ nAG 1pM (p<0.001), and astrocytes treated with TGFβ1+ nAG 1nM (p<0.001, Table II). The results of the nAG-only group as well as the TGFβ1+ nAG 100 nM group were not significantly different from controls (Table II).

For CSPG4, there was a significant increase in mRNA expression (compared to control) in three treatment groups: The TGFβ1 (p=0.006), the TGFβ1+nAG 1pM (p=0.003), and the TGFβ1+nAG 100 nM (p<0.001). This indicated that the effect of TGFβ1 is dominant over the effect of nAG (Table III). The results of the nAG-only group as well as the TGFβ1+ nAG 1 nM group were not significantly different from controls (Table III).

For S100B, there was a significant decrease in mRNA expression (compared to control) in four out of the five

**Table I:** BrdU proliferation assay (absorbance) of the treatment groups relative to control (the control result was considered as 100%).

| Treatment | Absorbance 1 | Absorbance 2 | Absorbance 3 | Average ±SD (**p-value) | *p-value |
|---|---|---|---|---|---|
| Control (no treatment) | 1.768 (100%) | 1.670 (100%) | 1.500 (100%) | 1.646 ±0.136 | 0.469 |
| nAG 1 nM | 1.406 (79.5%) | 1.410 (84.4%) | 1.414 (94.3%) | 1.41 ±0.004 (p = 0.371) | |
| TGFB1 | 1.779 (100.6%) | 1.520 (91.0%) | 1.352 (90.1%) | 1.55 ±0.215 (p = 0.999) | |
| TGFB1+ nAG1pM | 1.676 (94.8 %) | 1.406 (84.2 %) | 1.136 (75.7 %) | 1.406 ±0.27 (p = 0.861) | |
| TGFB1+ nAG1nM | 1.536 (86.9 %) | 1.534 (91.9 %) | 1.531 (102.1 %) | 1.533 ±0.002 (p = 0.830) | |
| TGFB1+nAG 100nM | 1.009 (57.1 %) | 1.200 (71.9 %) | 1.722 (114.8 %) | 1.310 ±0.369 (p = 0.818) | |

**By one-way analysis of variance (ANOVA), p was 0.469 indicating no significant difference between the groups.*
***By Post-Hoc (Dunnett T3 test), the control group was compared to each treatment group and none of the treatment groups showed a significant difference from control.*

**Table II:** Assessment of GFAP relative concentration of the treatment groups compared to control (the control result was considered as 1 and the results of all experimental groups were reported relative to control) .

| Treatments | Exp.1 | Exp. 2 | Exp.3 | Average ±SD | *p-value |
|---|---|---|---|---|---|
| Control | 1 | 1 | 1 | 1 ± 0.0 | <0.001 |
| nAG | 0.55 | 0.79 | 1.07 | 0.803 ±0.260 (p = 0.873) | |
| TGFβ1 | 0.11 | 0.06 | 0.08 | 0.083 ±0.025 (p = 0.001) | |
| TGFβ1+nAG 1pM | 0.11 | 0.08 | 0.09 | 0.093 ±0.015 (p <0.001) | |
| TGFβ1+nAG 1nM | 0.09 | 0.09 | 0.08 | 0.086 ±0.006 (p <0.001) | |
| TGFβ1+nAG 100 nM | 0.24 | 0.31 | 0.52 | 0.356 ±0.146 (p = 0.073) | |

**By one-way analysis of variance (ANOVA), P<0.001indicating a significant difference between the groups.*
***By Post-Hoc (Dunnett T3 test), the control group was compared to each treatment group. Three of the treatment groups showed a significant difference from control: The TGFβ1 (P=0.001), the TGFβ1+nAG 1pM (P<0.001), and the TGFβ1+nAG 100 nM (P<0.001).*

**Table III:** Assessment of CSPG4 relative concentration of the treatment groups compared to control (the control result was considered as 1 and the results of all experimental groups were reported relative to control).

| Treatments | Exp.1 | Exp. 2 | Exp.3 | Average ±SD (**p-value) | *p-value |
|---|---|---|---|---|---|
| Control (no treatment) | 1 | 1 | 1 | 1± 0.0 | <0.001 |
| nAG | 0.48 | 0.84 | 0.81 | 0.71 ±0.2 (p = 0.995) | |
| TGFβ1 | 4.22 | 2.83 | 3.82 | 3.62 ±0.716 (p = 0.006) | |
| TGFβ1+nAG 1pM | 4.22 | 2.78 | 4.36 | 3.78 ±0.875 (p = 0.003) | |
| TGFβ1+nAG 1nM | 3.44 | 1.97 | 3.16 | 2.86 ±0.781 (p = 0.058) | |
| TGFβ1+nAG 100 nM | 4.15 | 4.57 | 5.00 | 4.57 ±0.425 (p <0.001) | |

**By one-way analysis of variance (ANOVA), P<0.001 indicating a significant difference between the groups.*
***By Post-Hoc (Scheffe test), the control group was compared to each treatment group and three of the treatment groups showed a significant difference from control: The TGFβ1 (P= 0.006), the TGFβ1+nAG 1pM (p=0.003), and the TGFβ1+nAG 100 nM (p<0.001).*

**Table IV:** Assessment of S100 relative concentration of the treatment groups compared to control (the control result was considered as 1 and the results of all experimental groups were reported relative to control).

| Treatments | Exp.1 | Exp. 2 | Exp.3 | Average ±SD (**p-value) | *p-value |
|---|---|---|---|---|---|
| Control | 1 | 1 | 1 | 1 ±0.0 | <0.001 |
| nAG | 0.79 | 0.63 | 1.11 | 0.84 ±0.244 (p = 0.932) | |
| TGFβ1 | 0.22 | 0.24 | 0.28 | 0.25 ±0.031 (p = 0.002) | |
| TGFβ1+nAG 1pM | 0.28 | 0.26 | 0.24 | 0.26 ±0.02 (p = 0.001) | |
| TGF?1+nAG 1nM | 0.23 | 0.38 | 0.35 | 0.32 ±0.079 (p = 0.020) | |
| TGF?1+nAG 100 nM | 0.16 | 0.11 | 0.13 | 0.13 ±0.025 (p = 0.001) | |

**By one-way analysis of variance (ANOVA), P<0.001 indicating a significant difference between the groups.*
***By Post-Hoc (Dunnett T3 test), the control group was compared to each treatment group. With the exception of the nAG group, all other treatment groups were significantly different from control.*

**Table V:** Assessment for Interleukin 6 (IL-6) by using ELISA.

| NHA treatment | Absorbance (experiment 1) | Absorbance (experiment 2) | Absorbance (experiment 3) | Average ± SD (**p-value) | *p-value |
|---|---|---|---|---|---|
| Control (no treatment) | 2.389 | 2.949 | 3.595 | 2.978 ±0.604 | 0.115 |
| nAG 1 nM | 2.792 | 3.130 | 3.199 | 3.04 ±0.218 (p = 0.999) | |
| TGFB1 | 3.242 | 3.070 | 2.905 | 3.072 ±0.169 (p = 0.999) | |
| TGFB1+nAG 1 pM | 1.448 | 2.289 | 2.667 | 2.135 ±0.624 (p = 0.434) | |
| TGFB1+nAG 1 nM | 1.764 | 2.678 | 2.638 | 2.36 ±0.517 (p = 0.727) | |
| TGFB1+nAG 100 nM | 3.116 | 2.416 | 2.715 | 2.749 ±0.351 (p = 0.995) | |

**By one-way analysis of variance (ANOVA), P was 0.115 indicating no significant difference between the groups.*
***By Post-Hoc (Scheffe test), the control group was compared to each treatment group and none of the treatment groups showed a significant difference from control.*

treatment groups, *i.e.* the TGFβ1 (p=0.002), the TGFβ1+nAG 1pM (p=0.001), the TGFβ1+nAG 1 nM (p=0.020) and the TGFβ1+nAG 100 nM (p=0.001). The nAG-only group was not significantly different from control (p=0.932). This also indicated that the effect of TGFβ1 is dominant over the effect of nAG (Table IV).

There was a decrease in NG2 protein expression and inhibition of S100 protein in astrocytes treated with TGFβ1 (lane 2) and TGFβ1 + nAG 1nM (lane 3) compared to control astrocytes (lane 1) (Figure 1). There was more inhibition in NG2 protein expression in astrocytes treated with TGFβ1+ nAG than astrocytes treated with TGFβ1 alone (Figure 1). This result indicated the synergistic inhibitory effect of TGFβ1 and nAG on the expression of NG2 (CSPG4). IL-6 was measured in cell culture supernatant by ELISA and the results showed no significant difference (p=0.115) in IL-6 concentration in astrocytes with different treatments compared to control astrocytes as shown in Table V.

## DISCUSSION

Most previous molecular studies on reactive gliosis studied the profile of growth factors and inflammatory mediators as well as proteoglycan production from reactive astrocytes following an injury or secondary to a neurological disease.[8] Even in these injury or disease models, there is evidence that there is substantial heterogeneity of astrocyte response with regards gene/ protein expression and astrocyte cell morphology/ function depending on the type and extent of injury or disease.[8]

In this study, the authors studied the effects of TGFβ1 and nAG on normal 3-D astrocyte cultures, *i.e.* uninjured with no disease process. *In vitro* 3-D astrocyte cultures are known to be much less reactive compared to mono-layer and 2-D astrocyte cultures, but are thought to be a better mimic the normal central nervous system state.[7] Different concentrations of nAG were used in the treatment groups because low and high concentrations of nAG are known to be inhibitory and stimulatory to

fibrosis in human fibroblasts, respectively.[9] The results in the current experiment showed a general tendency of lack of significant changes with various doses in nAG (i.e. low, medium and high doses combined with TGFβ1 (Tables I-V). There were only two exceptions. The first exception was that the effect of TGFβ1+ nAG 100 nM on GFAP was different from other nAG groups (Table II); but the result in that group was not far from the other n AG groups with a p value of 0.07 (Table II). The second exception was that the effect of TGFβ1+ nAG 1 nM on CSPG4 was different from other nAG groups (Table III); but again the result in that group was also not far from the other nAG groups with a p value of 0.058 (Table III). The response to TGFβ1in previous studies has been incosistant,[1,3] and hence TGFβ1 was utilized in current experiment.

The study showed that neither TGFβ1 nor nAG had any effect on the proliferation of normal astrocytes. Following brain and spinal cord injury, there is increased astrocyte proliferation. The primary cause of this increased proliferation has been linked to fibroblast growth factor-basic (FGF-b) and interferon-gamma (IFN-γ) rather than TGFβ1.[10-12] This is interesting, because in the skin, TGFβ1 is the main mediator of fibroblast proliferation; and this effect is inhibited by nAG.[4] This emphasizes a major difference in the pathogenesis of skin fibrosis mediated by fibroblasts and central nervous system gliosis mediated by astrocytes.

One essential feature of reactive astrogliosis in injury and disease is the hypertrophy of its intracellular intermediate filament proteins. The most prominent astrocytic filament protein is the GFAP. In injury and disease states, TGFβ1 is known to be most important trigger for filament protein hypertrophy.[13-15] This study showed that TGFβ1 alone or with nAG will decrease (rather than increase) GFAP gene expression in normal astrocytes. Similarly, in the injured or diseased states, activated astrocytes produce excessive amounts of S100B protein. S100B protein is known to bind to Receptor for Advanced Glycation End-products (RAGE) which contributes to gliosis, oxidative stress, and neurotoxicity.[16] In contrast, this study showed that TGFβ1 alone or with nAG will reduce the production of S100B protein from astrocytes in the uninjured state. These findings indicate the presence of major differences between the effects on TGFβ1 on astrocytes in normal versus injury/disease states.

Following injury to the spinal cord, reactive astrocytes produce excessive proteoglycans in response to TGFβ1. The most prominent proteoglycans in this setting are the CSPG. These will physically block axonal regeneration and also indirectly inhibit axonal outgrowth by interfering with laminin (laminin is a growth promoter).[1] In an *in vivo* spinal cord crush injury model, nAG improved axonal regeneration and motor recovery; and also reduced the astroglyosis. This study in normal astrocyte cultures in-vitro showed very interesting findings regarding the effects of TGFβ1 and nAG on CSPG4. At the gene expression level, TGFβ1 alone or without nAG increased CSPG4 gene expression. However, TGFβ1 and one group of TGFβ1 and nAG reduced the protein expression of CSPG4. This brings the attention that an increased gene expression of CSPG4 may not always be associated with increased protein expression.

Finally, TGFβ1-induced astrogliosis in central nervous system injury and disease is known to be associated with increased production of the pro-inflammatory cytokine interleukin-6 (IL-6); which is also associated with increased proliferation of astrocytes.[17,18] These results showed that neither TGFβ1 nor nAG had an effect on IL-6 production in normal astrocytes. This is expected because neither TGFβ1 nor nAG had an effect on astrocyte proliferation.

One major issue that has rarely been mentioned in the literature of brain and spinal cord injuries is the significance of concurrent injury to the meninges. Injury to the axons *in vivo* will result in a pure astrocyte reaction. In contrast, injury to the axons and the meninges *in vivo* will result in a combined astrocyte-fibroblast reaction.[1] The latter fibroblast reaction is similar to the excessive fibrosis seen in the skin which is mediated with TGFβ1 and results in the excessive production of extracellular matrix proteins including collagen.[1] This may explain the differences in the results in injury models *in vivo* versus normal astrocyte culture models *in vitro*; including the lack of nAG effects in our culture model as compared to injury models.[6] It is now well established that TGFβ1 has major effects on cellular proliferation through the AKT-cellular proliferation pathway.[19] *In vivo* spinal cord injury (which has concurrent meningeal injury) is known to be associated with astrocyte proliferation which is linked to an increased TGFβ1 expression and its secondary effects on the AKT pathway.[20,21] These results of astrocyte proliferation in the *in vivo* spinal cord injury experiments help to clarify the role of the meningeal factor and also to clarify the differences between the previously reported *in vivo* studies compared to the current *in vitro* experiment regarding the results of astrocyte proliferation.

## CONCLUSION

The response of astrocytes at the gene level might not coincide with the response at the protein level. Furthermore, the effects of TGFβ1 were dominant over the effects of nAG with regard to changes in CSPG4 and S100B by real-time PCR. However, there was a synergistic inhibitory effect of TGFβ1 and nAG on the expression of CSPG4 by Western blot.

**Acknowledgement:** The project was funded by the College of Medicine Research Center, Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia.

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