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REGULATING THE AVAILABILITY OF TRANSFORMING GROWTH FACTOR β 1 IN B104 NEUROBLASTOMA CELLS

Amanda L. Lindke¹, Frank A. Middleton^{1,2}, and Michael W. Miller^{1,2,3}

¹ Department of Neuroscience and Physiology, State University of New York- Upstate Medical University, Syracuse NY 13210

² Developmental Exposure Alcohol Research Center, Binghamton NY 13902, Cortland NY 13045, and Syracuse NY 13210

³ Research Service, Syracuse Veterans Affairs Medical Center, Syracuse NY 13210

Abstract

Transforming growth factor (TGF) β 1 is a key player in early brain development, hence, its availability (i.e., synthesis and release) affects neuronogenesis. TGF β 1 moves proliferating cells out of the cell cycle and promotes their subsequent migration. The present study tested the hypothesis that neural progenitors self-regulate TGF β 1. B104 neuroblastoma cells which can grow in the absence of serum or growth factors were used in systematic studies of transcription, translation, release, and activation. These studies relied on quantitative enzyme-linked immunosorbant assays and real-time polymerase chain reactions. TGF β 1 positively upregulated its own intracellular expression and promoted increased release of TGF β 1 from cells. The induction of TGF β 1 was independent of a change in transcription, but it depended on cycloheximide-inhibited translation. Signaling mediated by downstream Smad2/3 through the TGF β receptors and intracellular protein transport were also required for release of TGF β 1 from B104 cells. Thus, TGF β 1 production and release was mediated through a feed-forward mechanism and was pivotally regulated at the level of translation. These activities appear to be key for the role of TGF β 1 in the proliferation and migration of young neurons.

Keywords

cytokine; development; Golgi complex; latency associated protein; neural stem cell; progenitor cell; proliferation; transcription; translation

INTRODUCTION

Transforming growth factor (TGF) β 1 is a multi-functional regulator of early brain development and subsequent maintenance. In the developing cerebral cortex, TGF β 1 pushes cells to exit the cell cycle (Luo and Miller, 1999; Siegenthaler and Miller, 2005) and then promotes the migration of young neurons in a concentration-dependent manner (Siegenthaler and Miller, 2004). The survival of post-mitotic, post-migratory neurons is also

Address mail to: Michael W. Miller, Department of Neuroscience and Physiology, SUNY- Upstate Medical University, 750 East Adams Street, Syracuse NY 13210, Ph. 315-464-7729; FAX 315-464-7725; millermw@upstate.edu.

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enhanced by TGF β 1 (Ren and Flanders, 1996; Tomoda et al., 1996; Brionne et al., 2003; Kuhn and Sarkar, 2008). As in the developing system, the growth- and survival-promoting effects of TGF β 1 are also evident in the mature cortex. TGF β 1 can modulate the proliferation of neural stem cells (NSCs; Battista et al., 2006; Goestaneh et al., 2006; Wachs, 2006) and boost neuronal survival (Makwana et al., 2007) in the adult. In the absence of TGF β 1, as in a conditional TGF β 1-null mouse, neuronal degeneration is widespread (Brionne et al., 2003), whereas TGF β 1 over-expression results in neuroprotection from mitochondrial toxin nitropropionic acid (Ueberham et al., 2005).

The effectiveness of TGF β 1 depends upon its bioavailability. The processes that underlie the availability of TGF β 1 are its synthesis (transcription and translation) and presentation in the extracellular milieu (secretion, activation, and clearance through degradation or receptor binding). Transcription at the TGF β 1 promoter is induced by agents such as growth factors, hormones, phorbol esters, and other cytokines both during normal development and in response to stimuli such as injury (Chang et al., 1999; Weigert et al., 2001; Miller and Luo, 2002a; Dhandapani et al., 2007). The efficiency of transcription differs among the three different TGF β transcripts (Wakefield et al., 1990; Arrick et al., 1991). These transcripts differ in the presence of a loop structure in the 5' region of the terminus. Cytosolic factors bind the 5' region and modulate translation efficiency (Romeo et al., 1993; Fraser et al., 2008).

TGF β 1 is initially synthesized as a propeptide (Miyazono et al., 1991). The propeptide is cleaved by enzymes, such as furin, to generate active TGF β 1 and a latency associated protein (LAP) (Dubois et al., 1995). LAP associates non-covalently with cleaved TGF β 1 and renders it unable to bind with TGF β receptor I (Wakefield et al., 1989; Miyazono et al., 1991). The latent TGF β 1 complex is constitutively released from the cell and activated extracellularly prior to receptor binding (Munger et al., 1997; Yehualaeshet et al., 1999). A key event in signal initiation is phosphorylation of a TGF β receptor (TGF β r; ten Dijke and Hill, 2004; Danielpour and Song, 2006). In this regard, it is important to note that the pool of TGF β 1 available for binding to a specific receptor(s) and initiating intracellular signaling is governed not by the total quantity of TGF β 1, but by the amount of TGF β 1 that is activated.

TGF β 1 can positively modulate its expression in non-neural cells (van Obberghen et al., 1988; Bascom et al., 1989; Liu et al., 1996; Zhang et al., 2006), astrocytes (Morgan-Kossmann et al., 1992), and microglia (Morgan et al., 2000). Thus, the present study used B104 neuroblastoma cells to test the hypothesis that TGF β 1 operates in cycling neuronal progenitors through a feed-forward regulatory system. As B104 cells behave like proliferating neuronal progenitors (cf. Luo and Miller, 1999a; Siegenthaler and Miller, 2005) and can differentiate into neurons, they provide insight into the role of TGF β 1 in normal brain development.

METHODS

Cell cultures

B104 neuroblastoma cells (ATCC, Manassas, VA) were derived from the rat central nervous system and raised in a serum-free medium (Schubert et al., 1974; Bottenstein and Sato, 1979). These cells were used for various reasons. (1) They display many of the markers and proliferative characteristics of neuronal progenitor cells (cf. Luo and Miller, 1999; Jacobs and Miller, 2001). (2) B104 cells can grow in a serum-free medium and in the absence of growth factors (Bottenstein and Sato, 1989; Luo and Miller, 1999). (3) B104 cells mimic neural progenitors in the cerebral ventricular zone which express both TGF β 1 and β 2 ligands and TGF β receptor (TGF β r) type I and type II (Miller, 2003). (4) The early development of B104 cells parallels that for cortical progenitors in two ways. (a) The

proliferation of B104 cells is inhibited by TGF β 1 (Luo and Miller, 1999) as it is for proliferating cells in the ventricular zone (Siegenthaler and Miller, 2005). (b) TGF β 1 affects the expression of downstream targets in B104 cells such as transcripts/proteins for adhesion molecules (Luo and Miller, 1999; Siegenthaler and Miller, 2004; Miller et al., 2006; Powrozek and Miller, 2009). Likewise, TGF β 1 also induces the expression of the same cell adhesion molecules in the cortical proliferative and migratory zones (Siegenthaler and Miller, 2004). (5) B104 cells can differentiate into excitable, neuron-like cells (Bottenstein and Sato, 1979).

B104 cells were maintained in Eagle's Minimal Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 33 mM glucose, and 100 U/ml penicillin/streptomycin (Gibco, Carlsbad, CA). The medium was changed every 2–3 days. Cells were incubated at 37°C in an atmosphere containing 6% CO₂ and saturating humidity. For most experiments, 1.20 x 10⁶ cells were plated in 100 mm Petri dishes and cultured overnight in the medium with FBS. The next day, cells were switched to a serum-free medium composed of equal parts Eagle's MEM and a solution composed of F12/HAM (Gibco), glucose, penicillin/streptomycin, 5.0 mg/l insulin, 5.0 mg/l transferrin, and 5.0 mg/l selenium (Sigma, St Louis, MO).

Treatment and sample collection

B104 cells were treated with TGF β 1 (0 or 1.0 ng/ml; R&D Systems, Minneapolis, MN) in serum-free medium for 0, 6.0, 24, or 48 hr. Following treatment, aliquots of medium were collected and frozen at –20°C and samples of cells were harvested. A single-cell suspension was produced by brief exposure to 0.25% trypsin with EDTA (Gibco). The lytic action of the trypsin was stopped with a serum-laced medium. A small aliquot of the cell suspension was reserved for cell counting. The numbers of viable cells were counted with a hemocytometer. Dead/dying cells, labeled with 0.20% trypan blue, were eliminated from the counts.

The remaining cells were centrifuged at 2000 rpm. The pellet was solubilized in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1.0% Tergitol, and Complete Mini-protease Inhibitor Cocktail (one tablet per 10 ml buffer; Roche, Indianapolis, IN) in sterile water; pH=7.6) and briefly sonicated prior to storage at –20°C.

Enzyme-linked immunosorbant assay

TGF β 1 concentrations in conditioned medium and cell lysate were determined using the E_{max} ImmunoAssay kit (Promega, Madison, WI). This kit used an antibody sandwich enzyme immunoassay technique to detect TGF β 1 with <2% cross-reactivity with TGF β 2 and TGF β 3. Specific antibodies to biologically active TGF β 1 were used; a monoclonal capture antibody and a polyclonal antibody made in rabbit. The assays detected concentrations of TGF β 1 as low as 30 pg/ml.

On the day of the assay, samples were thawed over ice and kit sample buffer (Promega) was added to stabilize the active TGF β 1. TGF β 1 concentrations were determined by comparison with a standard curve prepared by serial dilution of recombinant human TGF β 1 provided in the kit. TGF β 1 concentrations were normalized to the numbers of cells in a sample.

The concentration of total, active, and latent TGF β 1 were determined. The antibody in the ELISA kit was specific for active TGF β 1. Therefore, direct assays of medium and lysate samples were analyzed for active TGF β 1. Total TGF β 1 content was determined after an acidification step which converted latent TGF β 1 into the active form. Accordingly, samples were treated with 1.0 N HCl (pH<3.0) for 15 min and then returned to a neutral pH with 1.0 N NaOH before total TGF β 1 content was measured by ELISA. The amount of latent TGF β 1

was calculated as the difference between the total TGF β 1 content and amount of active TGF β 1 in each sample.

Transcript expression

B104 cells were cultured with or without TGF β 1 (10 ng/ml) for 3.0, 6.0, 12, 18, or 24 hr. Cultures were treated with trypsin (see above) to generate a cell suspension. The cells were pelleted by centrifugation at 2000 rpm. The pellet was rinsed twice in PBS and RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). The quality of the RNA was validated by comparison of the 28S:18S ratio with the Bioanalyzer RNA NanoChip (Agilent, Palo Alto, CA). RNA was quantified using UV spectrophotometry. Samples in which the ratio was >1.5 were considered acceptable for further processing.

The detailed procedure for the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) methods has been previously described (Miller et al., 2006). Briefly, expression for TGF β 1 transcript was determined by real time qRT-PCR relative to expression of an endogenous reference gene (18S ribosomal RNA) with the comparative C_T method. The mean difference in cycles to threshold between the target gene and the reference gene (ΔC_T) for each sample group ($\Delta\Delta C_T$) was used to calculate a fold change according to the formula ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen, 2001). qRT-PCR for TGF β 1 was performed on the Prism 7700 (Applied Biosystems, Foster City, CA). No bands were detected on gels when cDNA was excluded from the qRT-PCR.

Pharmacological studies

In some experiments, pharmacological reagents were added to the culture medium before, after, or during TGF β 1 treatment. These include blockers of protein translation and intracellular transport.

Cycloheximide (10 ng/ml in dimethylsulfoxide (DMSO); Sigma) was added as an inhibitor of protein translation. Two protocols were followed to resolve TGF β 1 content in the lysate and medium. (1) Cycloheximide was added to the serum-free medium 1.0 hr prior to TGF β 1 exposure. Fresh medium containing TGF β 1 (0 or 1.0 ng/ml) and either vehicle or cycloheximide was added for 1.0, 6.0, or 24 hr to cell cultures plated with 2.0×10^6 cells. (2) Cells were exposed first to TGF β 1 (0 or 1.0 ng/ml) for 24 hr. Vehicle or cycloheximide was introduced into the medium during the last hour of this incubation. Following a brief rinse, fresh medium with vehicle or cycloheximide was added for an additional 24 hr.

GolgiStop (active ingredient monensin; Becton-Dickinson, San Diego, CA) or GolgiPlug (active ingredient brefeldin A; Becton-Dickinson) were used as inhibitors of intracellular protein transport. Monensin blocked vesicular transport through the trans-Golgi network (TGN) to the plasma membrane, and brefeldin blocked transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Tartakoff, 1983; Lippincott-Schwartz et al., 1989; Dinter and Berger, 1998). In some experiments, cycloheximide (0 or 10 ng/ml in DMSO) was used in conjunction with the GolgiStop or GolgiPlug for simultaneous inhibition of protein translation and protein transport. Cells were cultured with TGF β 1 (0 or 1.0 ng/ml) for 24 hr. Vehicle or cycloheximide was introduced for the final hour of TGF β 1 exposure. This was fresh medium containing TGF β 1, (b) GolgiStop (0.375 μ l/ml) or GolgiPlug (0.40 μ l/ml), and (c) vehicle or cycloheximide. Samples of medium and cell lysates were collected six hours after adding the transport inhibitors.

TGF β r signaling is mediated via the phosphorylation of Smad2/3 (Heldin et al., 1997). Activation of Smad2/3 was inhibited by treatment with SB431542 (10 μ m in DMSO; VWR, West Chester, PA; Inman et al., 2002; Powrozek and Miller, 2009). Cells were cultured in

the presence of TGF β 1 (0 or 1.0 ng/ml) and vehicle or SB431542 in DMSO for 48 hr. At that time, samples of medium and cell lysates were collected for ELISAs.

Statistics

Quantitative data were assessed for statistical significance by two-way analyses of variance (ANOVAs). In situations where treatment or time was determined to be a significant ($p < 0.05$) factor, pair-wise comparisons were performed with a Holm-Sidak post-hoc test.

RESULTS

B104 cells secreted TGF β 1 and responded to exogenous TGF β 1

The number of B104 cells increased in dissociated cell cultures; it doubled every ~24 hr in a serum-free medium (Fig. 1). Cell number also increased in the presence of TGF β 1, however, this increase was significantly ($p < 0.01$) reduced relative to the controls at 48 hr.

Serum-free medium contained no detectable TGF β 1. Untreated cells released TGF β 1 into the medium at detectable concentrations within 6.0 hr post-plating (Fig. 2 top). The total concentration of TGF β 1 measured in the medium increased progressively, e.g., between 6.0 and 24 hr ($p < 0.01$) and continued between 24 and 48 hr ($p < 0.001$). Latent TGF β 1 was the primary form of TGF β 1 released into the medium. For example, latent TGF β 1 accounted for 71.5% of the total TGF β 1 detected at 48 hr. TGF β 1 concentration and cell number both increased with time. The concentrations of active and latent TGF β 1 were stable when normalized to respective cell counts (Fig. 2 bottom). Moreover, the ratio of active TGF β 1 to latent TGF β 1 in the medium did not vary over the time examined.

Addition of exogenous TGF β 1 to the culture altered the pattern of TGF β 1 expression and release. Addition of TGF β 1 significantly increased the concentration of active ($F_{1,39} = 134.332$; $p < 0.001$) and total ($F_{1,77} = 151.215$; $p < 0.001$) TGF β 1 content in the medium. Cell-derived active TGF β 1 was indistinguishable from exogenous TGF β 1 using the present ELISA protocol. On the other hand, addition of exogenous TGF β 1 did not directly contribute to the amount of latent TGF β 1 content in the medium; no latent TGF β 1 was present within 6 hr of when active TGF β 1 was added to the medium. The pattern of the decline of active TGF β 1 from the medium and the accumulation of latent TGF β 1 in the medium differed. Relative to the amount in the medium at the start of the experiment (1.0 ng/ml), the amount of active TGF β 1 expression declined between 6 and 24 hrs (Fig. 2 top). By 24 hr, only $\frac{1}{4}$ of the original amount of TGF β 1 was detected in the medium. Latent TGF β 1 concentration increased progressively between each time point, however, approximately 63% of the increase in latent TGF β 1 occurred between 24 and 48 hr. A similar pattern of early decline in active TGF β 1 and delayed accumulation of latent TGF β 1 occurred when TGF β 1 concentrations were normalized to cell counts (Fig. 2 bottom). Latent TGF β 1 concentration per cell increased significantly with respect to time ($F_{2,34} = 36.357$; $p < 0.001$) and treatment ($F_{1,34} = 16.929$; $p < 0.001$). The decline in active TGF β 1 and the increase in latent TGF β 1 were only evident in the cultures in which TGF β 1 was added.

Two conclusions can be drawn from the above data. (1) The net loss of $\frac{3}{4}$ of the active TGF β 1 in the medium resulted from the opposite actions of binding/degrading TGF β 1 and releasing new TGF β 1. Thus, more than 75% of the active TGF β 1 was degraded. This is supported by data examining the change in TGF β 1 concentration added to fresh medium in the absence of cells. The concentration of TGF β 1 fell precipitously after it was added to the medium (Fig. 3). More than $\frac{2}{3}$ of the TGF β 1 was lost during the first half hr, and less than 10% was detectable by 1.5 hr. (2) Latent TGF β 1 in the medium was not derived from the “reverse” conversion of active TGF β 1 to latent TGF β 1. Hence, the increase in the latent TGF β 1 derived from cell-generated TGF β 1.

TGFβ1 treatment corresponded to an increase in intracellular TGFβ1

The total TGFβ1 content in B104 cells prior to treatment was 14.3 ± 3.4 pg of TGFβ1 per million cells. This amount doubled during the first 24 hr post-plating in the control cultures and remained stable over the succeeding 24 hr (Fig. 4). The same pattern was evident for latent TGFβ1. It is important to note that latent TGFβ1 accounted for ~70% of cellular TGFβ1.

Addition of active TGFβ1 to the culture medium resulted in a significant increase in TGFβ1 (total ($F_{1,25} = 34.956$; $p < 0.001$), active ($F_{1,23} = 24.328$; $p < 0.001$), and latent ($F_{1,24} = 18.228$; $p < 0.001$) TGFβ1) in the cell lysate within 24 hr (Fig. 4). Internalized exogenous TGFβ1 likely contributed to the TGFβ1 content in the cell. The TGFβ1-induced increases in both forms of TGFβ1 were transient; they declined between 24 and 48 hr. Thus, treatment with exogenous TGFβ1 increased intracellular latent and active TGFβ1. TGFβ1 did not alter the proportion of cellular latent TGFβ1. The increase in intracellular latent TGFβ1 paralleled increases in latent TGFβ1 released to the medium.

TGFβ1 transcript expression was not altered by exogenous TGFβ1

Conceivably, changes in cellular TGFβ1 protein expression reflected changes in TGFβ1 transcript expression. The microarray study revealed no apparent changes in transcript expression between 6 and 18 hr. This was verified by qRT-PCR for temporal changes in TGFβ1 transcript (Fig. 5). No significant differences caused by the TGFβ1 treatment or time were detected using ANOVAs. This was supported by fitting the data to lines by a least squares regression method. The data for the control cultures were fit to a line wherein $r^2 = 0.794$ ($p < 0.05$). Accordingly, there was a slow increase in transcript content with time. On the other hand, the regression analysis revealed that TGFβ1 had no detectable effect on the expression of TGFβ1 mRNA during 24 hr of treatment.

Translation of TGFβ1 was important for cellular auto-induction by TGFβ1

Cycloheximide, added to B104 cells prior to TGFβ1 treatment, abolished the TGFβ1-induced of latent TGFβ1 in cell lysate (Fig. 6) at each timepoint where induction of latent TGFβ1 was observed. The TGFβ1-induced increase in active TGFβ1 was not abrogated by cycloheximide, however, the inductive effect of TGFβ1 was significantly ($F_{3,46} = 33.991$; $p < 0.001$) blunted as evident within one hour of TGFβ1 treatment. Thus, cycloheximide profoundly inhibited the synthesis of latent TGFβ1. At the same time, cycloheximide had a minimal effect on active TGFβ1 content within the cell.

To determine if the TGFβ1 release into the medium following induction was translation-dependent, the TGFβ1 content of conditioned medium and cell lysates was evaluated in the presence of cycloheximide following TGFβ1 exposure. The potential conundrum of differentiating exogenous TGFβ1 from endogenously generated TGFβ1 released by the B104 cells was avoided by exposing the cultures to TGFβ1 prior to cycloheximide treatment. This medium was replaced with a serum-free medium containing cycloheximide and the TGFβ1 content of the medium was measured after an additional 24 hr of culture.

Cells with previous exposure to active TGFβ1 released significantly ($F_{1,28} = 52.054$; $p < 0.001$) more latent TGFβ1 in the 24 hr following removal of TGFβ1 (Fig. 7 top). The release of latent TGFβ1 was abolished by cycloheximide. Cycloheximide also significantly ($F_{2,26} = 112.959$; $p < 0.001$) reduced the cellular content of latent TGFβ1 (Fig. 7 bottom). This was congruent with the effect of cycloheximide pretreatment on the cellular content of latent TGFβ1 (cf. Fig. 4). In the presence of cycloheximide, there remained a significant ($p < 0.001$) TGFβ1-induced difference in latent TGFβ1 content. Thus, a portion of intracellular latent TGFβ1 was refractile to cycloheximide.

Active TGF β 1 release into the medium was significantly ($p < 0.001$) increased when vehicle (DMSO) was present in cultures with prior exposure to TGF β 1. Cycloheximide did not affect active TGF β 1 release into the medium. Prior TGF β 1 exposure significantly ($F_{1,30} = 21.16$; $p < 0.001$) increased intracellular active TGF β 1 content and was not affected by cycloheximide.

Intracellular vesicular transport is required for TGF β 1 release

Inhibiting translation prevented accumulation of latent TGF β 1 in conditioned medium. This implied that protein transport from the Golgi apparatus to the cell membrane was required for latent TGF β 1 accumulation in medium. To confirm the requirement for intracellular transport in TGF β 1 accumulation, the effect of inhibiting protein transport on TGF β 1 production and release was evaluated. Following a 24 hr treatment with TGF β 1, medium was replaced with medium containing GolgiPlug or GolgiStop. Both treatments abolished latent TGF β 1 from the medium (Fig. 8 top). This confirmed that intracellular vesicular transport was required for latent TGF β 1 to be present in the medium.

Inhibiting secretion did not affect the amount of active TGF β 1 in the medium. Twenty-four hours of prior exposure to TGF β 1 significantly ($F_{1,38} = 39.014$; $p < 0.001$) increased the medium content of active TGF β 1 on a per cell basis in the first six hours following TGF β 1 removal. Blocking protein transport with GolgiStop or GolgiPlug, either alone or in combination with cycloheximide treatment, had no effect on active TGF β 1 in the medium. Thus, translation and intracellular transport of TGF β 1 did not contribute to the detection of active TGF β 1 in the medium.

The absence of latent TGF β 1 in conditioned medium in the presence of GolgiStop was expected to cause an accumulation of intracellular latent TGF β 1. Assays of latent TGF β 1 content in lysates of cells exposed to GolgiStop or GolgiPlug showed that intracellular latent TGF β 1 accumulation did not occur (Fig. 8 bottom). Instead, the concentration of intracellular latent TGF β 1 in the presence of GolgiStop was not statistically different from cells with no exposure to the secretory inhibitor. Treatment with GolgiPlug decreased the TGF β 1-induced intracellular TGF β 1 content ($p < 0.001$). Addition of cycloheximide to the treatment medium in combination with GolgiStop, but not GolgiPlug, significantly ($p < 0.01$) decreased latent TGF β 1 in cell lysate.

Signaling through TGF β r and Smad2/3 was necessary for TGF β 1 auto-induction

TGF β 1-mediated activity requires the ligand to bind to a specific receptor and successful TGF β r binding is evidenced by the phosphorylation of Smad2/3. Following inhibition of TGF β 1-induced Smad2/3 signaling, there was a sharp decline (80.5%, $F_{2,29} = 80.767$; $p < 0.001$) in latent TGF β 1 accumulation in the medium (Fig. 9 top). The amount of basal latent TGF β 1 secreted into serum-free medium also decreased (56%, $p < 0.01$) when Smad2/3 signaling was inhibited. This was consistent with the concept of TGF β 1 auto-induction. In contrast, active TGF β 1 concentration in the medium was not affected when Smad2/3 signaling was inhibited.

Although TGF β 1 auto-induction was inhibited by blocking Smad2/3 signaling, latent TGF β 1 was still evident in the medium. This indicated that part of the cellular response to TGF β 1 was independent of Smad2/3 signaling or not specific to the TGF β r. Inexplicably, cellular latent TGF β 1 was not sensitive to inhibition of Smad2/3 signaling (Fig. 9 bottom). Thus, there was an apparent dissociation between TGF β 1 content in the medium which depended upon translation, secretion, and Smad2/3 signaling, and TGF β 1 content of the cell lysate which was partially or wholly independent of these same processes.

DISCUSSION

Role of TGF β 1 in B104 cell proliferation

TGF β 1 inhibits the proliferation of B104 cells. A similar response is evident in other neural cells, e.g., astrocytes (Vergeli, 1995; Miller and Luo, 2002a), C6 glioma cells (Miller and Luo, 2002a), neuronal progenitors (Miller and Luo, 2002b), and NSCs in dissociated (Hicks et al., submitted) and organotypic cultures including the cerebral ventricular zone (Siegenthaler and Miller, 2005). At least in the explants of the cerebral wall, the anti-proliferative effect results from TGF β 1-promoted cell cycle exit (Siegenthaler and Miller, 2005). The decreases in cell proliferation occur in the absence of TGF β 1-induced cell death (Luo and Miller, 1999; Miller and Luo, 2002b).

The inhibitory effects of TGF β 1 are concentration-dependent. For example, the proliferation of B104 cells is moderately inhibited (19%) by exposure to relatively low concentrations of TGF β 1 (1.0 ng/ml) (present study). Following exposure to a higher concentration (10 ng/ml), growth inhibition is trebled (59%) (Lou and Miller, 1999). Concentration-dependent responses are also evident for TGF β 1-promoted migration of cortical cells (Siegenthaler and Miller, 2004). Accordingly, TGF β 1 maximally stimulates migration at a concentration of 10 ng/ml and has an attenuated effect at 1.0 ng/ml. Thus, the present study used an effective, but submaximal concentration of TGF β 1.

B104 neuroblastoma cells are capable of autocrine/paracrine regulation. They respond to TGF β 1, as evidenced by a TGF β 1-induced reduction in cell growth (noted above), and they produce and release TGF β 1. B104 cells express both TGF β 1 transcript and protein and TGF β r. Intracellular endogenous TGF β 1 is detectable in both latent and active forms by ELISA; latent TGF β 1 is the predominant form accounting for 76% of all TGF β 1.

Production of TGF β 1

Intracellular expression of active and latent TGF β 1 increase following exposure to TGF β 1. The increase in intracellular latent TGF β 1 results from the synthesis of newly generated TGF β 1 not from the uptake of extracellular TGF β 1. The relative contributions of newly generated TGF β 1 and uptake from the extracellular pool of TGF β 1 are not differentiated by immunoassay. When TGF β 1 is added to a cell-free medium, there is rapid degradation of the TGF β 1 so that within 1.5 hr, less than 10% remains. Nevertheless, the increase in intracellular latent TGF β 1 following TGF β 1 exposure is blocked by prior exposure to the protein synthesis inhibitor, cycloheximide. This implies that TGF β 1 up-regulates its own expression. Xxx degradation study also add Hicks ref

Unexpectedly, TGF β 1-induction of intracellular and released latent TGF β 1 protein from B104 cells is not accompanied by a corresponding auto-induction of TGF β 1 mRNA expression. The qRT-PCR data are consistent with microarray data from TGF β 1 treated B104 cells that show no auto-induction or change in gene expression for any TGF β 1 isoform with TGF β 1 treatment. Auto-induction of TGF β 1 mRNA precedes the release of TGF β 1 into the medium in several non-transformed and transformed cell types (van Obberghen et al., 1988; Flanders et al., 1995; Liu et al., 1996; Morgan et al., 2000). The promotor region which binds the transcription factor AP-1 is thought to mediate auto-induction of the TGF β 1 message (Kim et al., 1990).

Protein up-regulation independent from transcript expression is indicative of post-transcriptional regulation. Consistent with TGF β 1-induced post-transcriptional modulation of TGF β 1 protein synthesis, inhibition of *de novo* protein synthesis with cycloheximide blocks TGF β 1 induction in B104 cells.

Post-transcriptional activities are key events in TGF β 1 regulation. Evidence for this comes from studies showing that the expression of TGF β 1 mRNA and protein are not always correlated. For example, peripheral blood monocytes express TGF β 1 mRNA, but they do not synthesize TGF β 1 protein until activated (Assoian et al., 1987). Discrepancies between TGF β 1 mRNA and protein expression have been described in other cultured cells (Glick et al., 1989; Wakefield et al., 1990; Kim et al., 1992; Steigerwald, 1992). Additionally, a study in HK-2 renal cells shows that insulin-induced increases in TGF β 1 protein expression occur with no effect on TGF β 1 mRNA (Morrisey et al., 2001).

The presence of a G-C rich stem loop structure in the 5' UTR of the TGF β 1 mRNA regulates translational efficiency of TGF β 1 transcripts. This process is mediated through binding of cytosolic proteins and allows for post-transcriptional regulation of TGF β 1 protein (Kim et al., 1992; Romeo et al., 1993; Allison et al., 1998). Thus, TGF β 1 signaling in B104 cells might regulate cytosolic enhancer proteins to promote TGF β 1 translation and expression.

Release of intracellular stores of TGF β 1

B104 cells release TGF β 1 into culture medium and most of the TGF β 1 detectable in the medium is in the latent form. These data contrast with an earlier study showing that B104 cells only secrete active TGF β 1 (Asakura et al., 1997). The reasons for the discrepancy are not clear, because almost all cells primarily secrete latent TGF β 1. Relative comparison of TGF β 1 release between cell types is confounded by a lack of normalization in reporting of ELISA concentrations relative to a fixed quantity such as the number of cells present. Data from human fetal lung fibroblasts suggests comparable release to that seen in this study; approximately 2 ng TGF β 1 per million cells within 48 hr (Wen et al., 2003).

TGF β 1 promotes the intracellular expression and release of latent TGF β 1 from B104 neuroblastoma cells. Latent TGF β 1 detected in the medium does not result from conversion of exogenous TGF β 1 to a latent state. The pattern of rapid decline in active TGF β 1 and delayed accumulation of latent TGF β 1 in the medium argues against direct conversion. Moreover, signaling through TGF β r-activated Smad2/3 is required for expression of latent TGF β 1 in the medium. Augmentation of TGF β 1 protein release following TGF β 1 treatment depends on vesicular transport from the Golgi apparatus to the cell membrane. Both GolgiPlug and GolgiStop block the release of TGF β 1 protein from B104 cells. The presence of latent TGF β 1 in the medium depends on TGF β r signaling through Smad2/3, protein translation, and intracellular protein transport.

The present study does not determine what portion, if any, of the latent TGF β 1 released by B104 cells is activated and contributes to signaling through the endogenous TGF β 1 system. B104 cells in serum-free medium release latent TGF β 1, but they do not exhibit TGF β 1 feed-forward induction. This suggests that activation of latent TGF β 1 generates an insufficient concentration of active TGF β 1 to achieve a signaling threshold for TGF β 1-induction. The concentration of active TGF β 1 required to induce TGF β 1 amplification cannot be determined from the current data which did not examine the effects of TGF β 1 concentrations below 1.0 ng/ml.

Interestingly, the GolgiPlug (brefeldin A) and GolgiStop (monensin) differentially affect intracellular concentrations of TGF β 1. GolgiPlug, but not GolgiStop, reduces intracellular TGF β 1 and blocks TGF β 1 augmentation in B104 cells. A possible explanation for these data derives from differential disruption of protein processing and cleavage of pro-TGF β 1. GolgiPlug disrupts vesicular transport between the endoplasmic reticulum and the *cis*-Golgi (Lippincott-Schwartz et al., 1989), whereas GolgiStop disrupts protein transport through the TGN (Tartakoff, 1983). Speculatively, GolgiPlug disrupts protein transport prior to cleavage of pro-TGF β 1 by furin in the TGN (Dubois et al., 1995; Schafer et al., 1995) and pro-TGF β 1

is not recognized by the ELISA antibody to active mature TGF β 1. Evidence for brefeldin A, and not monensin, disrupting furin cleavage comes from the study of cleavage of a proliferation-inducing ligand in human embryonic kidney 293T cells (Lopez-Fraga et al., 2001) and β -amyloid processing (Sudoh et al., 1998).

Conceivably, blocking TGF β 1 protein release should result in intracellular accumulation of TGF β 1 protein. This does not occur. No further augmentation beyond that seen with TGF β 1 treatment alone is detected with either transport inhibitor. The implication is that intracellular TGF β 1 accumulation is negatively regulated, perhaps through a TGF β 1 signaling inhibitor such as Smad7.

Feed-forward regulation

Multiple signaling pathways can mediate TGF β 1 up-regulation. TGF β 1 signaling through TGF β r potentially activates Smad2/3, ERK1/2, and pathways downstream of p38 with some degree of cross-talk (Luo and Miller, 1999; Mulder, 2000; Javelaud, 2005; Danielpour and Song, 2006; Powrozek and Miller, 2009). Smad2/3 and ERK1/2 signaling are critical for the auto-induction of TGF β 1 mRNA in renal proximal tubular epithelial cells, whereas inhibitors of p38 (mitogen-activated protein kinase) selectively blocked induction of TGF β 1 protein synthesis without affecting TGF β 1 mRNA expression (Zhang, 2006). In B104 cells, the TGF β 1-induced release of TGF β 1 into culture medium is a direct result of signaling through Smad2/3 intermediates; TGF β 1 release is blocked by Smad2/3 inhibition. This is distinct from intracellular TGF β 1 which is not affected by Smad2/3 inhibition and requires other signaling intermediates. The connection between Smad2/3 signaling and regulation of cytosolic factors which modify TGF β 1 translation is currently unknown.

The behavior of proliferating B104 cells and stem/progenitor cells in the developing cerebral cortex are similar (Luo and Miller, 1999; Miller and Luo, 2002a; Siegenthaler and Miller, 2004; 2005; Miller et al., 2006). Moreover, the response of the B104 cells to TGF β 1 is like that of proliferating cells in dissociated cell cultures and organotypic cultures of the fetal cerebral wall *in situ*. Thus, the present results with B104 cells provide insight into the role of the feedback system in cell proliferation and migration during cortical development *in vivo*.

Radial glia express a TGF β ligand and receptor (Miller, 2003). These cells serve two roles during cortical development. (1) They are neural stem cells (Malatesta et al., 2000; Noctor et al., 2001, 2002; Gotz et al., 2002) and (2) they provide the physical scaffold used by young, post-mitotic neurons to migrate into the cortical plate (Rakic, 1972). Both cell proliferation (Siegenthaler and Miller, 2005) and neuronal migration (Siegenthaler and Miller, 2004) in the cerebral wall are affected by TGF β 1. Thus, TGF β 1 is strategically expressed by a population of cells that is involved in TGF β 1-regulated activities.

The self-stimulatory action of TGF β 1 is involved in altering the proliferation and migration in the developing cerebral wall. Initially, the cortical plate consists primarily of proliferating progenitor cells with few cells exiting the cell cycle into post-proliferating populations (referred to as the quiescent or leaving fraction) (Miller and Kuhn, 1995; Takahashi et al., 1996). Formation of the laminar architecture of the cerebral cortex occurs as an increasing number of cells enter into the quiescent fraction. TGF β 1 is a candidate for controlling the progressive shift in balance from a proliferating to a post-proliferating cell population (Siegenthaler and Miller, 2005). Presumably, the initial TGF β 1 released by radial glia stimulates feed forward autocrine/paracrine TGF β 1 synthesis. Progressive amplification of TGF β 1 signaling results in a consequent increase in cells exiting from the cell cycle and entering the post-proliferative population.

Migration of cells along radial glial fibers following the cessation of proliferation is a second activity promoted by increasing concentrations of TGF β 1 (Siegenthaler and Miller, 2004). TGF β 1 ligands and receptors are of greatest expression in the central segment of the radial glial fibers (i.e., the part distributed in the proliferative and intermediate zones) (Miller, 2003). These zones are the sites where the initiation and propagation of migration, respectively, occur. Thus, feed-forward TGF β 1 production would not only drive cells to exit the cell cycle, it would also promote robust migration of these same cells along radial glial fibers. As a sufficient number of young neurons is generated and stimulated by TGF β 1 to migrate to form the cortical layers, continued amplification of TGF β 1 serves as a self-regulating termination signal since migration is inhibited by high concentrations of TGF β 1 (Siegenthaler and Miller, 2004).

In summary, B104 cells both express and respond to TGF β 1. TGF β 1 promotes its own intracellular expression and subsequent release into the extracellular environment by a post-transcriptional mechanism that does not involve the auto-induction of TGF β 1 transcription. The accumulation of TGF β 1 protein in the extracellular environment depends on Smad2/3 signaling and intracellular protein transport, whereas other signaling pathways underlie intracellular TGF β 1-induction. Thus, TGF β 1 self-promotes its own intracellular expression and subsequent release into the extracellular environment. This sets up a positive feed-forward autocrine/paracrine system whereby the physiologic effects of TGF β 1 on proliferation and migration can be progressively amplified.

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The abbreviations used are

ANOVAs	analyses of variance
CNS	central nervous system
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
FBS	fetal bovine serum
LAP	latency associated protein
qRT-PCR	quantitative real-time polymerase chain reaction
TGF	transforming growth factor
TGF β r	transforming growth factor β receptor
TGN	trans-Golgi network

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Figure 1. Effects of TGFβ1 on the numbers of B104 cells

The number of cells in untreated cultures (solid bars) increased over time. Treatment with TGFβ1 (1.0 ng/ml; open bars) impeded this increase. Each value is the mean of three to five samples ($\pm$ the standard error of the mean). Statistical significance is denoted by *, #, and @ for differences relative to the controls at the same time, to samples at the same condition at 6 hr, and to samples at the same condition at 24 hr, respectively.

Figure 2. TGFβ1 content in the medium

The amount of TGFβ1 (total and active) was determined using quantitative ELISAs. Latent TGFβ1 content was calculated as the difference between the concentrations of total and active TGFβ1. Cells were raised in a serum-free medium (solid bars) or treated with the same medium supplemented with TGFβ1 (1.0 ng/ml; open bars). The upper set of graphs describes the concentration of TGFβ1 per ml medium, whereas the lower triad of graphs depicts the same data in terms of TGFβ1 concentration per million cells. The number of cells in the cultures varied over time and was affected by TGFβ1 treatment. Therefore, the concentration of TGFβ1 was calculated in relation to the number of cells in the sample as depicted in the lower graphs. Notations as in Figure 1. n = 4 to 6.

Figure 3. TGFβ1 degradation

The change in the concentration of TGFβ1 over time was traced in medium without cells. Each datum is the mean of four samples ($\pm$ the standard error of the mean).

Figure 4. TGFβ1 content in the cell lysate

TGFβ1 content following TGFβ1 exposure (1.0 ng/ml) was determined by ELISA and expressed relative to the number of cells present. Notations as in Figure 1. n = 3 to 5.

Figure 5. TGFβ1 transcript expression

The effect of exogenous TGFβ1 (1.0 ng/ml) on the expression of its own transcript was determined using qualitative real-time polymerase chain reaction. Changes in TGFβ1 transcript were normalized to expression of 18S ribosomal RNA and expressed as in terms of fold change relative to the amount of transcript in 3 hr control. A best-fit line was generated by linear regression. n = 3 to 5.

Figure 6. Prior cycloheximide exposure inhibits TGFβ1-induction in cell lysates

The TGFβ1 content of cell lysates was quantified by ELISA and normalized to cell counts. One hour of exposure to vehicle or cycloheximide (10 ng/ml in DMSO) was followed by timed exposure to TGFβ1 (0 or 1.0 ng/ml) with continued vehicle or cycloheximide exposure. Statistically significant differences are identified by the following symbols: * for differences relative to the controls as the same time, % for differences relative to the samples treated with TGFβ1 at the same time, and # and @ for similarly treated samples at the 1 hr and 6 hr timepoints, respectively. n = 3 to 5.

Figure 7. Cycloheximide blocks TGFβ1-induced latent TGFβ1 release

Following 24 hr of exposure to TGFβ1 (0 or 1.0 ng/ml), B104 cells were treated for an additional 24 hr to serum-free medium (none), vehicle (DMSO), or cycloheximide (10 ng/ml). The TGFβ1 content of conditioned medium (top) and cell lysates (bottom) was quantified and normalized to cell counts. Each * denotes a statistically significant ($p < 0.05$) difference relative to control for the same treatment. A # identifies a difference relative to cells raised in a serum-free condition. An @ signifies a difference relative to samples exposed to the vehicle DMSO. $n = 3$ to 6.

Figure 8. Intracellular transport inhibitors block TGFβ1 release

Cells were exposed to TGFβ1 (1.0 ng/ml) for 24 hr followed by a 6 hr exposure to protein transport inhibitors (GolgiStop and GolgiPlug) with or without cycloheximide (10 ng/ml). TGFβ1 content of the medium (top) and cell lysate (bottom) were determined by ELISA. An * denotes a statistically significant ($p < 0.05$) difference relative to control for the same treatment. A # identifies a difference relative to samples exposed to the vehicle DMSO. An @ signifies a difference relative to GolgiStop or GolgiPlug for the same condition. A % designates a significant difference for the comparison of GolgiStop or GolgiPlug for the same condition. $n = 3$ to 6.

Figure 9. TGFβ1-induced TGFβ1 release requires TGFβr signaling through Smad2/3

TGFβ1 content of the medium (top) and cell lysate (bottom) were assayed following 48 hr of simultaneous exposure to TGFβ1 (0 or 1.0 ng/ml) and the Smad2/3 inhibitor SB431542 (0 or 10 μm). Notations as in Figure 6. n = 4 to 6.