Dickkopf 1 Mediates Glucocorticoid-Induced Changes in Human Neural Progenitor Cell Proliferation and Differentiation

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Received August 19, 2011; accepted October 20, 2011

Glucocorticoids (GC) are critical for normal development of the fetal brain, and alterations in their levels can induce neurotoxicity with detrimental consequences. Still, there is little information available on the effects of GC on human neural stem/progenitor cells (hNPC). In the present study, we have investigated the effects of the synthetic GC dexamethasone (Dex) on hNPC grown as neurospheres, with special focus on their proliferation and differentiation capacity and the underlying molecular mechanisms. Immunocytochemical stainings showed that Dex markedly decreases proliferation and neuronal differentiation while promoting glia cell formation. Analysis of pathway-specific genes revealed that Dex induces an upregulation of the Wnt-signaling antagonist DKK1. Moreover, Dex- or DKK1-treated hNPCs showed reduced transcriptional levels of the two canonical Wnt target genes cyclin D1 and inhibitor of DNA binding 2 (ID2). Chromatin immunoprecipitation showed that Dex, via the glucocorticoid receptor, interacts with the DKK1 promoter. Treatment of hNPC with recombinant DKK1 or neutralizing antibodies indicated that DKK1 has a critical role in the Dex-induced inhibition of proliferation and neuronal differentiation with a concomitant increase in glial cells. Taken together, our findings show that GC reduce proliferation and interfere with differentiation of hNPCs via the canonical Wnt-signaling pathway.

Key Words: glucocorticoids; Dickkopf 1; Wnt signaling; human neurospheres; proliferation; differentiation.

Neural stem cells (NSCs) give rise to the major cell types of the brain, i.e., neurons, astrocytes, and oligodendrocytes. They are present in the ventricular neuroepithelium of the developing brain and in discrete neurogenic areas in the adult brain where they represent a unique cell population with neurogenerative capacity (Eriksson et al., 1998). Maintenance and differentiation of NSC are regulated by intrinsic and extrinsic signaling cascades, including the Wnt pathway. Wnt signaling is involved in the early patterning of the neuroectoderm and has an essential role in promoting proliferation during the expansion of the NSC pool. Wnts are also involved at later stages of neural development during differentiation and lineage decisions, as well as in neurite growth and axon guidance (Ille and Sommer, 2005). Wnt signals are transmitted via several pathways (Nusse et al., 2008). In the canonical pathway, Wnts bind to frizzled and low-density receptor-related protein (LRP) 5/6 complexes, which activates downstream cascades leading to phosphorylation of disheveled, inactivation of glycogen synthase kinase (GSK)3β, and inhibition of β-catenin phosphorylation, which consequently inhibits degradation of intracellular β-catenin. Accumulated β-catenin translocates to the nucleus and activates target genes by a complex formed with T-cell factor (TCF)/lymphoid enhancer family transcription factor. Canonical Wnt signaling is inhibited by the secreted Dickkopf (DKK) glycoproteins. DKK1 negatively modulates the canonical Wnt-signaling pathway due to binding and subsequent endocytosis of the co-receptor LRP5/6.

Dysregulation of Wnt signaling has been implicated in many pathological conditions, and increased levels of DKK1 have been linked to the neurodegenerative processes that precede neuronal death in experimental models of excitotoxicity and β-amyloid toxicity (Caraci et al., 2008).

The development of most organs, including the nervous system, is influenced by hormones, such as glucocorticoids (GC). The action of GC is mediated by its interaction with the glucocorticoid receptor (GR) or the mineralocorticoid receptor, which transactivate or transrepress transcription of target genes after stimulation via GC binding. Regulation of gene expression by the GR can occur in two distinct ways: (1) the ligand-activated GR interacts directly with specific DNA sequences, defined as GC-responsive elements (GREs) that are present in target genes and (2) GR-mediated stimulation of gene expression takes place by GR interaction with other DNA-bound proteins without direct binding to the DNA itself or by interacting with other molecules involved in intracellular signaling (Beck et al., 2009). Treatment with synthetic GC, including dexamethasone (Dex), has become common practice to prevent the serious complications associated with preterm birth, successfully reducing early
neonatal death (Liggins and Howie, 1972). However, several human studies have shown that treatment with synthetic GC can have detrimental effects on the developing brain with subsequent behavioral alterations and cerebral palsy (Baud, 2004; Bodensteiner and Johnsen, 2005; French et al., 2004; Murphy et al., 2001). In addition, there is growing evidence from experimental studies that there may be lifelong effects on neuroendocrine functions following exposure to excess GC (Kapoor et al., 2008). Elevated GC levels decrease cell proliferation in the dentate gyrus in various mammalian species (Ambrogini et al., 2002; Gould et al., 1997; Kim et al., 2004; Wong and Herbert, 2005), whereas removal of endogenous steroids by adrenalectomy increases neuronal cell birth (Cameron and Gould, 1994). It has also been shown that steroids by adrenalectomy increases neuronal cell birth. So far, there is little information available on the effects of GC on human neural stem/progenitor cells (hNPCs). Therefore, the present study was designed to investigate the effects of a synthetic GC, Dex, on hNPC proliferation and differentiation and characterizes the underlying molecular mechanisms. As an experimental model, we used cultures of primary neurospheres isolated from human fetuses. The results show that Dex act through DKK1 that (1) mediates GC-induced inhibition of neurosphere proliferation and (2) promotes GC-stimulated cell differentiation toward the glial cell type with a concomitant decrease in neurons.

MATERIALS AND METHODS

Chemicals. Dex and mifepristone (RU486) were obtained from Sigma-Aldrich. Recombinant human DKK1, neutralizing anti-human DKK1, and mouse IgG isotype control antibodies were purchased from R&D Systems.

Cell culture. Human NPC (Lonza Verviers SPRIL) from three different individuals (gestational weeks 16, 16.5, and 19) were cultured as neurospheres as previously described (Moors et al., 2010). Briefly, spheres were cultured in DFB medium (Dulbecco’s Modified Eagle’s Medium and Ham’s F12 [2:1] supplemented with B27 [Invitrogen], 20 ng/ml EGF [Invitrogen], and 20 ng/ml rhFGF [R&D Systems]) at 37°C with 5% CO2.

hNPC treatments. hNPC grown as neurospheres with a diameter of 0.25–0.3 mm were plated on poly-o-lysin/laminin–coated 10-cm dishes (1 × 106 cells) and stimulated with 1μM Dex or solvent control for 90 min. Subsequently, formaldehyde was added (1%, 10 min). The reaction was stopped by freezing (0.125M, 5 min). After washing, cells were resuspended in 400 μl lysis buffer (50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1mM EDTA, 0.5% Tween 20, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride [PMSF]) containing Complete Protease Inhibitor Cocktail (Roche Applied Science). Chromatin was sheared (fragments around 500 bp–1 kb) by sonication for 15 min using a Diagenode Biozoom. Subsequent concentrations of chromatin-containing supernatants obtained after centrifugation were incubated overnight with 2 μg anti-GR antibody (sc-8992; Santa Cruz Biotechnology, Santa Cruz, CA) and crosslinked with 1% formaldehyde for 10 min. Experiments were performed on cells from the different individuals in triplicates.

Proliferation/differentiation analyses. For proliferation assays, five to six spheres per donor were exposed in DFN medium supplemented with 20 ng/ml EGF (Invitrogen) and 20 ng/ml rhFGF (R&D Systems) for 4 h followed by immunocytochemical analyses. Differentiation assays were performed with five to six neurospheres per donor in DFN without growth factor supplementation for 4 days before immunocytochemical analyses.

Immunocytochemistry. hNPC were fixed in 4% paraformaldehyde for 30 min. After rinsing and blocking in 0.5% bovine serum albumin for 1 h, neurospheres were stained with antibodies against the proliferation marker Ki67 (1:1,000; Novocastra Laboratories Ltd.), the NSC marker nestin (1:200; BD Biosciences), the neuronal marker TuJ1 (1:200; Covance), the glia marker glial fibrillary acidic protein (GFAP, 1:400; Dakocytomation), or with the oligodendroglia marker 2’,3’-cyclic nucleotide 3’-phosphodiesterase (CNPase, 1:200; Sigma) overnight at 4°C. As secondary antibodies, we used Alexa 488 and Alexa 594 (1:300; both from Invitrogen Molecular Probes). Nuclei were stained with Hoechst 33342 (0.1 μg/ml; Sigma-Aldrich) to determine total cell numbers. Slides were examined with a fluorescent microscope (Olympus) and photographed with a digital camera (Hamamatsu C4742-95; Hamamatsu). Proliferation and differentiation were quantified by counting the number of positive cells within the 2D migration area around the sphere core in three different fields per sphere.

Gene expression analyses. Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions including on-column DNase digestion (RNase-free DNase Set; Qiagen) for 30 min at 25°C. Complementary DNA (cDNA) was synthesized from equal amounts of RNA by using Superscript II First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer’s protocol. Product accumulation was measured by real-time PCR analyses based on SYBR Green detection via ABI Prism 7000 Sequence Detection System with SDS software (version 2.1; Applied Biosystems, Foster City, CA). Expression levels were normalized to the housekeeping genes β-actin and hypoxanthine phosphoribosyltransferase (HPRT) (ΔcT = cT (target gene) – cT (housekeeping gene)), which showed no Dex-induced changes in gene expression (data not shown). Relative expression levels were calculated as ΔΔcT = ΔcT (Dex) – ΔcT (control) and expression changes were calculated as 2−ΔΔcT. PCRs contained 1 μl cDNA, 0.2μM of each primer, and SYBR Green PCR Master Mix (Applied Biosystems; amplification protocol: 10 min 95°C [AmpliTaq Gold DNA Polymerase activation]; 15 s 95°C, 1 min 56–60°C [40 cycles]). Primer sequences and annealing temperatures used for quantitative real-time PCRs are listed in Supplementary table 1. Product specificity was determined via melting curve analyses (temperature ramp from 60°C to 95°C) and agarose gel electrophoresis. All experiments were done on three replicate samples from at least two cell preparations from the different individuals.

Chromatin immunoprecipitation assay. For chromatin immunoprecipitation (ChIP) analysis, dissociated hNPC were plated on poly-o-lysin/laminin–coated 10-cm dishes (1 × 106 cells) and stimulated with 1μM Dex or solvent control for 90 min. Subsequently, formaldehyde was added (1%, 10 min). The reaction was stopped by freezing (0.125M, 5 min). After washing, cells were resuspended in 400 μl lysis buffer (50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1mM EDTA, 0.5% Tween 20, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride [PMSF]) containing Complete Protease Inhibitor Cocktail (Roche Applied Science). Chromatin was sheared (fragments around 500 bp–1 kb) by sonication for 15 min using a Diagenode Biozoom. Subsequent concentrations of chromatin-containing supernatants obtained after centrifugation were incubated overnight with 2 μg anti-GR antibody (sc-8992; Santa Cruz Biotechnology, Santa Cruz, CA).
Dex Interferes with hNPC Proliferation and Differentiation

Immunocytochemical analyses of hNPC cultured with EGF and FGF showed that Dex exposure reduces the number of Ki67-positive cells as compared with controls (Figs. 1A–C). The percentage of Ki67-positive cells after exposure to Dex was significantly decreased (50.5 ± 6% compared with untreated controls, p < 0.05). Analyses with markers for NSCs (nestin), glia cells (GFAP), and neurons (Tuj1) showed that Dex interferes with hNPC spontaneous differentiation resulting in a significant decrease of cells positive for nestin (50 ± 7% of controls, p < 0.05) (Figs. 1D–F) or Tuj1 (47 ± 5% of controls, p < 0.05) (Figs. 1G–I) and a concomitant increase in the number of cells positive for GFAP (60 ± 10% of controls, p < 0.05) (Figs. 1J–L). No CNPase-positive cells were detected either in control or in Dex-treated cells.

DKK1 Is a Primary Target of the GR

Wnt family members are glycoproteins that are involved in the control and maintenance of various stem cell types (Nusse et al., 2008). We first concentrated on Dex-induced effects on the Wnt-signaling cascade. Frizzled (FZZD) or catenin like (CTNNL) showed no differences in expression, whereas there was a significant upregulation of the Wnt antagonist DKK1 (Fig. 2A). No changes were observed in DKK2, 3, and 4. To further investigate the mechanisms of Dex-stimulated DKK1 induction, we pre-exposed neurospheres to the GR antagonist mifepristone (RU486). Mifepristone inhibited the Dex-induced
FIG. 2. Dex alters the expression of DKK1 via GR interaction. (A) Neurospheres were exposed to 1μM Dex for indicated time under differentiating conditions (control, Con; black, Dex; gray). (B) Neurospheres were pre-exposed to 1μM mifepristone (Mife) for 30 min before exposure to Dex (1μM) for 24 and 48 h. Experiments were performed on two different cell preparations in quadruplicates for each preparation and reproduced at least twice. Relative expression levels were normalized to the housekeeping gene β-actin. Changes in expression were calculated as 2^{-ΔΔCT} with ΔΔCT = ΔCT_Dex − ΔCT_Control (ΔCT = CT (target gene) − CT (housekeeping gene). Values are shown as mean ± SEM. Statistical significance was determined by two-way ANOVAs in combination with a Bonferroni post hoc test (*significant vs. controls; #significant vs. Mife).

Increase of DKK1 messenger RNA pointing to a GR-dependent mechanism. After 24 h, Dex led to a 2.3 ± 0.1-fold DKK1 induction, which was reduced to 1.4 ± 0.1-fold by mifepristone. Similar results were detected after 48 h (Fig. 2B).

To determine whether the GR directly interacts with the DKK1 promoter, we performed a ChIP analysis. The DKK1 promoter contains three putative GREs within 1 kb from the transcription start site (Fig. 3A). Therefore, we selected primers adjacent to these GREs for amplification reactions (Fig. 3A). Exposure to Dex followed by precipitation with a GR-specific antibody resulted in a significant 3.5-fold enrichment of the corresponding sequence located in the last exon of the DUSP1 gene (negative control) served as controls. Experiments were performed in a one cell preparation and reproduced three times. Values are shown as mean ± SEM. Statistical significance was determined by two-way ANOVAs in combination with a Bonferroni post hoc test (p < 0.05; *significant vs. controls; #significant vs. Mife).

DKK1 Mediates Dex-Induced Alterations in Human Neurosphere Proliferation and Differentiation

Next, we investigated whether DKK1 expression is involved in Dex-induced alterations in proliferation and differentiation capacity of hNPC. Figure 4 shows that recombinant DKK1 mimics Dex-induced changes in proliferation (Fig. 4A) and differentiation (Figs. 4B and 4C); incubation of neurospheres with DKK1 protein results in a significant decrease in Ki67-positive cells (Fig. 4A). Furthermore, DKK1 treatment leads to a decrease of TuJ1-positive cells (Fig. 4B) with a concomitant increase in the number of GFAP-positive cells (Fig. 4C) as compared with controls. DKK1 is a secreted protein, which can be blocked by neutralizing antibodies. Coexposure to an anti-human DKK1 antibody and Dex blocked Dex-induced alterations in proliferation (Fig. 4A) and differentiation (Figs. 4B and 4C). In contrast, the isotype control antibody had no effects (Figs. 4A–C). All together, these data indicate that Dex-induced alterations are regulated by an increased expression of DKK1.

Dex and DKK1 Exposure Affects Expression of Canonical Wnt-Signaling Targets

DKK1 is a well-established negative regulator of the canonical Wnt-signaling pathway. We therefore analyzed the effects of GC or DKK1 exposure on known Wnt targets. Exposure to Dex for 24 h led to a decrease in non-phosphorylated β-catenin, although with a slight variability among the samples (data not shown). Cyclin D1 and ID2 are direct targets of canonical Wnt signaling, with effects on stem cell proliferation and maintenance (Panhuysen et al., 2004; Willert et al., 2002). Neurospheres exposed to 1μM Dex or 300 ng DKK1 showed reduced levels of Cyclin D1 and ID2 expression as measured by real-time PCR (Figs. 5A and 5B), suggesting that Dex and DKK1 regulate hNPCs via the canonical Wnt-signaling pathway.
In light of the compelling evidence of developmental neurotoxic effects exerted by GC, including the synthetic forms, in this study, we have investigated the effects of Dex on proliferation and differentiation of hNPC. Our data show that the Wnt antagonist DKK1 is a major target for Dex and a critical mediator of the observed Dex-induced alterations in neural proliferation and differentiation. That GC mediate disturbances in fetal and adult neurogenesis by interfering with stem cell proliferation has been established in several in vitro and in vivo experimental models, including rat embryonic NSC (Bose et al., 2010; Kim et al., 2004; Lemaire et al., 2000; Sundberg et al., 2006).

**DISCUSSION**

Wnt signaling is known to positively regulate maintenance and proliferation of different types of stem cells, and it has been suggested that its proliferative action is mainly mediated by the canonical Wnt/β-catenin pathway (Michaelidis and Lie, 2008; Nusse et al., 2008). In respect to NSCs, for example, Wnt3-mutant mice showed decreased proliferation, whereas Wnt3 overexpression led to enhanced neurogenesis in the hippocampus (Lee et al., 2000; Lie et al., 2005). Overexpression of Wnt1 in the developing mid/hindbrain resulted in enhanced proliferation and induction of the positive cell cycle regulator cyclin D1, a known Wnt-signaling target (Panhuysen et al., 2004). Also, stable β-catenin overexpression has been shown to have a dramatic effect on proliferation in the developing mouse cortex (Chenn and Walsh, 2002). DKK1 is a negative regulator of canonical Wnt signaling and has been found to inhibit NSC colony formation from single cells in vitro by inhibiting endogenous Wnts (Kalani et al., 2008). These data are in agreement with our results showing that the Dex dependent decreased proliferation of hNPCs occurs via induction of DKK1 and reduced Wnt signaling. Our findings indicate that GC also interferes with the spontaneous differentiation process, induced by growth factor withdrawal, thereby inhibiting neuronal differentiation while promoting the formation of glial cells. These results are in line with data from animal models providing evidence that GC induce organizational changes in distinct regions of the brain (Matthews, 2001; Welberg and Seckl, 2001) and link GR-induced transcriptional actions to an inhibition of neuronal differentiation. One of the current hypotheses for GC-mediated changes in neurogenesis is based on the idea that the decreased formation of neurons is due to the reduced proliferation of NSC, without effects on differentiation (Bose et al., 2010; Elder et al., 2006; Kim et al., 2004). Our present findings showing Dex-induced inhibition of neuronal differentiation associated with an increased glia formation clearly show that in hNPC differentiation is also...
affected by GC. The decrease in the number of neuronal cells was not due to increased apoptosis since the concentration of Dex used in our studies did not cause cell death at any time, as stated in the “Materials and Methods” section.

In contrast to our data, it has been reported that Dex selectively inhibits astroglia differentiation of rat midbrain NSC (Sabolek et al., 2006), as shown by a decreased number of GFAP-positive cells. In addition to species-related differences, variations in culture protocols may also provide an explanation for the discrepancy in the results; we examined the effects on hNPC after 4 days of spontaneous differentiation in the absence of the growth factors EGF and FGF in an N2-supplemented medium to evaluate early alterations in differentiation induced by exposure to Dex. In contrast, rat midbrain NSCs were differentiated for 14 days without N2-supplementation (Sabolek et al., 2006). Considering that glia cells belong to mitotic cell types, fewer GFAP-positive cells might be due to a Dex-induced inhibition of glia cell proliferation, as previously reported (Crossin et al., 1997). In addition to methodological differences, the specific characteristics of the neural stem/precursor cells of different origin and timing might explain the discrepancies between the above-mentioned studies and ours.

The identification of DKK1 as a primary target for the GR in human neurospheres provides a mechanistic-based explanation for the GC-induced alterations during neurogenesis. Stimulation with recombinant DKK1 protein showed that DKK1 inhibits proliferation and promotes differentiation toward the glia phenotype. A DKK1-induced increase in gliogenesis has also been reported for mouse NSC. DKK1 treatment of neurospheres derived from neonatal mouse forebrains caused inhibition of canonical Wnt signaling seen by luciferase TCF reporter gene assay and robustly increased the gliogenesis at the expense of neurogenesis (Kunke et al., 2009). Experimental animal models have also shown that neurogenesis is severely reduced in the hippocampal dentate gyrus in DKK1 transgenic mice (Solberg et al., 2008). A correlation between DKK1 expression and proliferation has also been observed in human glioblastoma, where epigenetic silencing via hypermethylation of the DKK1 gene was found in 50% of secondary glioblastomas (Götze et al., 2009).

A GC-mediated inhibition of Wnt-dependent transcription has been suggested as an indirect mechanism by which GC activate GSK3β with subsequent decrease in β-catenin levels (Kassel and Herrlich, 2007). Apart from its function in the canonical Wnt pathway, DKK1 also has β-catenin–independent functions via c-Jun N-terminal kinase (JNK) activation and subsequent blocking of Wnt-LRP6 signaling. DKK1 has been reported to interfere with the noncanonical JNK/Wnt pathway in Ewing tumor cells that exhibit neuronal features (Endo et al., 2008). Furthermore, Lee et al. (2004) provided strong evidence for the antagonizing effect of DKK1 on Wnt signaling through the JNK/Wnt pathway, which points to a crosstalk between the canonical and noncanonical JNK/Wnt pathway upstream of β-catenin.

In light of the fact that NSCs are present not only in the fetal but also in the adult brain, where they represent a resident population of cells that maintain throughout life neurogenerative capacity (Eriksson et al., 1998), our findings might be relevant not only in relation to neurodevelopment but also to adult physiological and pathological conditions. Of additional concern are the recent data showing that the GC-induced effects on NSCs are heritable and likely occur via epigenetic changes (Bose et al., 2010). Interestingly, emerging evidence suggests that decreased Wnt signaling promotes age-related defects and cellular aging (DeCarolis et al., 2008). Recent findings have pointed to an increased expression of DKK1 causally related to neurodegenerative processes associated with Alzheimer’s disease or brain ischemia (Rosi et al., 2010). DKK1 expression was found to be increased in cultured neurons challenged with β-amyloid peptide in vitro and in neurons showing signs of degeneration in brain tissue from Alzheimer’s patients (Caricasole et al., 2004). Cultured cortical fetal rat neurons exposed to DKK1 showed decreased canonical Wnt signaling seen by TCF reporter and β-catenin levels, loss of BCL-2, induction of BAX, and increased cell death (Scali et al., 2006). Zhang et al. (2008) found that the neuroprotective role of estrogen during hippocampal ischemic insults in rats is largely due to its capacity to prevent elevation of DKK1. Moreover, inhibition of the Wnt pathway has been implicated in mood disorders, such as depression (Gould et al., 1997). Interestingly, the expression of the neurotrophic factors VEGF and BDNF, alleged to be involved in the pathology of depression, is also regulated by Wnt signaling (David et al., 2008; Pishvaian and Byers, 2007).

All together, our data support the hypothesis that the Wnt-signaling pathway is critical in mediating the effects of Dex on hNPC and suggest that similar mechanisms may be involved in the GC-exerted neurotoxicity in the developing human brain.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

**FUNDING**

Swedish Research Council (project numbers 21379 and 10815).

**ACKNOWLEDGMENTS**

We thank Dr Ellen Fritsche for donating the hNPC and Dr Roshan Tofighi and Dr Ola Hermanson for critical reading of the manuscript. The authors confirm that there are no conflicts of interest.


