Species-dependent differences of embryonic stem cell-derived neural stem cells after Interferon gamma treatment

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INTRODUCTION

Neural stem cells (NSCs) are multipotent and can give rise to the three principle cell types found in the adult mammalian brain, namely neurons, astrocytes, and oligodendrocytes. In vivo, they can be found in the hippocampus and subventricular zone where they mainly give rise to new neurons in the dentate gyrus and olfactory bulb, a process called neurogenesis (Kempermann, 2002, 2011). In vitro, they can be kept under proliferative conditions exhibiting immature neural markers like nestin and Sox2 due to the stimulus of growth factors, or differentiate to the above mentioned mature cell types when growth factors are withdrawn (Temple and Qian, 1996; Temple, 2001). Various regenerative approaches are aimed at using the capacity of NSCs as a therapeutic agent by transplanting them into injured brain regions (Barker et al., 2003; Harrower and Barker, 2005; Gogel et al., 2011). Additionally, NSCs from various sources are increasingly used to simulate diseases in vitro in order to set up model systems that can be easily manipulated and investigated. A specific and important advantage of in vitro model systems for neurological diseases is the possibility to use a human genetic background on the basis of human pluripotent stem cells (pSCs) in order to exclude, for instance, murine-specific phenomena. One interesting question, that could be clarified in vitro under controlled conditions, is how inflammatory processes that consistently occur in various neurological diseases act on NSCs. As in vivo neurogenesis is more and more accepted to contribute to processes like, for instance, memory function (Deng et al., 2009, 2010), it appears to be of interest to characterize effects of pathological processes such as inflammation on NSCs. Inflammation itself may lead to damage in brain tissue and represents either a primary disease entity or a secondary phenomenon following (Campbell, 2005), for instance, cerebral ischemia (Whitney et al., 2009). Interferon gamma (IFNγ), a pro-inflammatory key player, is a cytokine that is secreted by various cell types such as cytotoxic CD8+ T-cells, natural killer cells (Griffin, 1997), astrocytes, fibroblasts, and endothelial cells (Rady et al., 1995; De Simone et al., 1998; Wei et al., 2000). IFNγ signaling takes place via the IFNγ receptor which consists of two chains, situated in the cell membrane with an extra- and intracellular part (Schreiber and Farrar, 1993). The structure and the amino acid sequence of the murine and the human IFNγ protein and its receptor differ, although the physiological function remains the same (Farrar and Schreiber, 1993). These structural differences are leading to species-specific IFNγ—IFNγ receptor interactions with human IFNγ affecting only human and other primate cell types and vice versa (Schreiber et al., 1992; Schroder et al., 2004). IFNγ receptors were found on murine NSCs and therefore, effects of IFNγ on murine NSCs and related alterations in neurogenesis in vivo (Kim et al., 2002; Lin et al., 2004; Sweeten et al., 2004; Wang et al., 2004, 2008) and in vitro (Kim et al., 2007; Makela et al., 2010) were excessively...
explored. However, only little is known about the response of human NSCs (hNSCs) to IFNγ. We previously investigated effects of IFNγ on murine embryonic day 14-derived stem/precursor cells (msNSPCs) and murine embryonic stem cell-derived NSCs in vitro (Walter et al., 2011, 2012) (both populations are referred to as mNSCs in the following text). Predominantly in proliferative mNSC cultures, we found that IFNγ leads to a dysregulated phenotype, characterized by synchronous expression of neuronal and glial markers despite the presence of growth factors. This was accompanied by an unusual electrophysiological phenotype on single cell level preventing the ability to form synchronously bursting functional neuronal networks after differentiation. We also demonstrated an IFNγ-related significant up-regulation of sonic hedgehog (SHH) and Stat 1, key down-stream signals that are important for induction of the above mentioned phenotype (Walter et al., 2012). To assess the relevance of these findings with respect to the human situation, we (1) treated human embryonic stem cell-derived hNSCs with this pro-inflammatory cytokine and (2) measured IFNγ concentrations in cerebrospinal fluid (CSF) specimens collected from patients suffering from different nervous system diseases.

RESULTS

hNSCs EXPRESS IFNγ RECEPTOR I AND II

In a first step, we immunocytochemically characterized the hNSC population generated from immature pluripotent embryonic stem cells. After neural pre-differentiation, almost all cells expressed nestin and most cells (>80%) expressed both, nestin and Sox2 (Figure 1A). After withdrawal of bFGF, NSCs terminally differentiated into βIII-tubulin+ neurons or GFAP+ astrocytes (Figure 1A). As the expression of membrane-bound IFNγ receptors (2 IFNγ receptor-1 chains and 2 IFNγ receptor-2 chains) is necessary to transduce the IFNγ signal, we performed immunocytochemical labelings against both receptor chains and were indeed able to demonstrate their expression (Figure 1B). We further investigated the expression levels of both receptor chains on mRNA level by means of real-time quantitative PCR. We compared these data with those generated on a murine background and found, that murine and human NSCs did not show significant differences (Figure 1C). Data were generated with the Wicell h9 line. We also confirmed these findings by using the HUES 6 line (data not shown).

hNSCs DO NOT EXPRESS THE DYSREGULATED PHENOTYPE AFTER IFNγ EXPOSURE

One major characteristic of the IFNγ-induced mNSC dysregulation is the coexpression of neuronal and glial markers under the influence of growth factors that normally hold NSCs in an immature and proliferating state (Figure 2A). This phenomenon is visible after a 3-days treatment with 1000 U/ml IFNγ and leads to a portion of around 39% of all cells that co-express GFAP and βIII-tubulin. The detailed characterization of this phenomenon is published elsewhere (Walter et al., 2011). However, when immature and proliferating hNSC populations were cultured with human recombinant IFNγ in identical concentrations compared to the murine situation, we were not able to detect this phenomenon (Figure 2A). These

FIGURE 1 | hNSC express IFNγ-R1 and IFNγ-R11. In (A) representative photomicrographs of undifferentiated and differentiated hNSCs (Wicell h9 line) are given. To characterize the undifferentiated stage, proliferating cells under the influence of growth factors were seeded and then immediately PFA fixed for immunocytochemistry. To characterize the differentiated stage, cells were seeded and differentiated for four weeks with an initial stepwise reduction of growth factors. In the upper panel immunocytochemical stainings against the neural precursor markers nestin and Sox2 in undifferentiated hNSCs are given demonstrating the pre-differentiation protocol which starts with pluripotent stem cells yields populations highly enriched in neural precursor cells. In the lower panel immunocytochemical stainings against βIII-tubulin and GFAP in differentiated hNSCs are given demonstrating the terminal differentiation of hNSCs into neurons and astrocytes. In (B) representative photomicrographs of undifferentiated hNSCs (Wicell h9 line) are given. A co-immunocytochemical staining against nestin and IFNγ-R1 is given in the upper panel and against nestin and IFNγ-R11 in the lower panel showing that both IFNγ receptor subunits are expressed in undifferentiated hNSCs. In (C) gene expression levels of IFNγ-R1 and IFNγ-R11 in undifferentiated hNSCs (Wicell h9 line) and mNSCs is shown.
results were also confirmed on mRNA level (Figure 2B). Data were generated with the Wicell H9 line. We also confirmed these findings by using the HUES 6 line (data not shown).

**IFNγ-REGULATED GENES IN hNSCs AND EFFECTS OF IFNγ ON THE POPULATION SIZE OF hNSCs**

As previously reported, the genes for inducible nitric oxide synthase (i-NOS) (Komatsu et al., 1996), IRF-9 (Ousman et al., 2005), c-Myc (Ramana et al., 2000), major histocompatibility complex 1 (MHC 1) (Johansson et al., 2008) and Stat 1 (Lehtonen et al., 1997) are significantly regulated in murine cells after IFNγ exposure. We now investigated the expression of these downstream signaling genes after IFNγ exposure in hNSCs by means of real-time quantitative PCR. We found all of these genes to be significantly up-regulated after IFNγ exposure also in hNSCs (Figure 3A). As previously reported, we also found SHH to be significantly up-regulated in mNSCs after IFNγ treatment. Interestingly, we could not detect that phenomenon in hNSCs. Another IFNγ-induced phenomenon on a murine background was a strong reduction of the population size of undifferentiated mNSCs. However, we were not able to detect a significant decrease in the population size of hNSCs after IFNγ exposure (Figure 3B). Data were generated with the Wicell H9 line. We also confirmed these findings by using the HUES 6 line (data not shown).

**IFNγ IS UP-REGULATED IN THE CEREBROSPINAL FLUID OF PATIENTS SUFFERING FROM DIFFERENT DISEASES**

In contrast to the extensively described up-regulation of IFNγ in animal models of human central nervous system diseases, there is only very limited information about amounts of IFNγ in human individuals suffering from neurological diseases. Therefore, we measured the concentration of IFNγ in cerebrospinal fluid (CSF) specimens, taken after lumbar punction from patients suffering from multiple sclerosis, peripheral nervous plexus affections, Alzheimer dementia, viral myelitis, or stroke. We found, that patients suffering from multiple sclerosis during an acute relapse showed a significant increase of IFNγ (three control samples vs. three MS samples). Due to the limitation of patient material, we could only screen single patients with other diseases (one patient per other indicated disease). But also here we found a clear trend toward an up-regulation of IFNγ in the CSF (Figure 3C).

**DISCUSSION**

We found here that IFNγ has species-dependent effects on embryonic stem cell-derived neural populations. While this pro-inflammatory key player induces a striking dysregulation in undifferentiated murine neural stem cells (mNSCs) with an unusual coexpression of neuronal and glial markers (Walter et al., 2011, 2012), it has no such effects on human NSCs although both cell populations express appropriate IFNγ receptors and up-regulate most of the classical down-stream signals like i-NOS, IRF-9, c-Myc, MHC 1, and Stat 1, which are known to mediate important IFNγ effects (Schroder et al., 2004).

One possible explanation for this diverse reaction of murine or human NSCs toward IFNγ exposure might be subtle differences in their developmental state or brain region-specific differentiation although both populations consist of proliferating nestin- and Sox2-positive neural precursors. However, the differentiation protocols used in this study were similar for both species leading to a heterogeneous population of neural cells without preference of distinct brain-region specific phenotypes (Illes et al., 2009; Lappalainen et al., 2010; Yla-Outinen et al., 2010). To account for developmental differences, we tested additional IFNγ treatment paradigms for human NSCs including a 7-days treatment or a 5-fold IFNγ concentration but could not detect an up-regulation of neuronal or glial markers or even the appearance of cells that synchronously express these markers (data not shown).
Another explanation for the divergent reactions after IFN-γ exposure might be a qualitatively different induction of downstream signals. While SHH is significantly up-regulated after IFN-γ treatment in mNSC populations, it remains unaltered in hNSC populations. Interestingly, recently published results of our group show that the IFN-γ-induced dysregulated murine phenotype depends on both, Stat 1 and SHH signaling (Walter et al., 2012). Thus, the lack of an important down-stream signal in the human situation might explain the species-dependent differences. As it is known that human gliomas and tumorigenic NSCs express SHH (Eltshem et al., 2007) and comprise cells that simultaneously express neuronal and glial markers (Katsetos et al., 2001; Ignatova et al., 2002; Singh et al., 2003; Walton et al., 2009), this morphogene was associated with brain tumor formation and/or growth. Interestingly, in mice a link between IFN-γ and SHH signaling was observed as an ectopic expression of IFN-γ was shown to induce medulloblastoma formation via SHH overexpression (Lin et al., 2004). Our results in general point to the fact that, possibly fundamental differences in cytokine-induced signaling pathways between human and mouse lead to significant differences in the development of cellular phenotypes. Nevertheless, this type of fundamental differences can also be observed within a single genetic background if, for instance, merely the developmental age of a given cellular population differs. In this regard, several studies investigated the role of IFN-γ in human lymphocyte activation and found that, depending on their developmental age (neonatal vs. adult); IFN-γ induces divergent down-stream signaling pathways leading to significant differences in their ability to respond to pathogens (Wilson et al., 1986; Marodi et al., 1994, 2001; Marodi, 2002). Discussed mechanisms for this divergent response of neonatal or adult lymphocytes to IFN-γ are a less effective Stat 1 phosphorylation in neonatal cells or an up-regulation of a new class of cytokine signaling suppressors that can inhibit JAK-Stat pathway (Endo et al., 1997; Starr et al., 1997). In summary, our results admittedly reduce the relevance of an IFN-γ-induced dysregulation of undifferentiated NSCs in a human genetic background. However, regarding the above mentioned considerations, it appears to be possible that human NSCs of a different developmental stage in comparison to those we used here can still react to IFN-γ exposure with an even misguided initiation of differentiation programs. Thus, our results can rather be interpreted in a way that a given cellular population in vitro, even on a human genetic background, might overemphasize results as it not reflects the complexity of an organism. This consideration is substantiated by observations from Johansson et al. (2008). In their study, immortalized hippocampal or striatal human neural stem/progenitor cells from 12-weeks-old fetal brains showed increased neurogenesis and MHC 1 expression after IFN-γ exposure during their differentiation phase without growth factors. The divergent results in comparison to our study might simply be explained by the developmental stage of the population during IFN-γ exposure as we used proliferating human NSCs under the influence of growth factors. That human fetal cells are principally able to coexpress GFAP and βIII-tubulin under non-inflammatory conditions in the ventricular and subventricular zones as well as under culture
conditions was shown by Draberova et al. (2008). Probably this geno- and phenotypic rare case is only possible in a specific time frame of human fetal development and independent from inflammatory stimulation.

To further substantiate the relevance of IFNγ during human CNS diseases, we verified the up-regulation of IFNγ in the CNS. We demonstrate here that patient CSF contains elevated amounts of IFNγ in comparison to control CSF of healthy individuals. Significantly elevated IFNγ levels were found during relapses in multiple sclerosis (n = 3) and we also found increased levels in individual patients suffering from peripheral nervous plexus affections, Alzheimer dementia, viral myelitis, or stroke (n = 1, respectively). This is in accordance with the fact that human peripheral lymphocytes can secrete up to approximately 200–500 U/ml IFNγ under neuroinflammatory conditions (Hirsch et al., 1985; Chan et al., 1991). As brain ependymal cells lack tight junctions, the CSF compartment in the brain ventricles exchanges neuroactive substances with the interstitial fluid of the brain parenchyma, including neurogenic zones (Alvarez-Buylla and Lim, 2004; Shen et al., 2008; Tavazoie et al., 2008; Ming and Song, 2011; Hartung and DiMone, 2012). This might point to a possible anatomical relationship between IFNγ accumulation within the CSF and NSCs within neurogenic zones, substantiating the relevance of IFNγ effects on NSCs in humans. In summary, our results demonstrate that data collected on a murine genetic background cannot automatically be translated to the human situation and that even on a human background results might differ depending on the developmental stage of the population and their maturation.

MATERIALS AND METHODS

CELL CULTURE

The human NSCs used in this study were either derived from the human embryonic stem cell lines HUES 6 (hESC facility; Harvard University; Cambridge, MA, USA) or purchased in a neuroectodermal, predifferentiated form of the human embryonic stem cell line H9 (WiCell Research Institute, Madison, WI, USA/Invitrogen, Minneapolis, USA), or purchased in a neuroectoder-

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For immunocytochemistry, cells were seeded on coated cover slips (VWR International, Darmstadt, Germany). After 3 days under the influence bFGF (20 ng/ml both Tebu-bio) and under different treatments as indicated, the cells were fixed with 4% PFA (Roti-Histofix, Carl Roth, Karlsruhe, Germany) for 15 min at room temperature. Cells were blocked for 30 min at room temperature with 1 fold Roti-Immu-no-Block containing 0.25% Triton X-100 for cell wall permeabilization (Carl Roth, Karlsruhe, Germany). Primary antibodies used at 4°C overnight were against βIII-tubulin (Tuji; 1:500; R&D Systems, Minneapolis, USA), glial fibrillary acid protein (GFAP) (1:500; Dako, Hamburg, Germany), Sox2 (1:50; R&D Systems, Minneapolis, USA), IFNγ-R1 (1:500 Santa Cruz Biotechnology, Heidelberg, Germany), IFNγ-R2 (1:500; Santa Cruz Biotechnology, Heidelber, Germany) and nestin (1:200; Covance, Munich, Germany). For detection of primary antibodies, fluoresceine-isothiocyanate (FITC; 1:500; Millipore, Billerica, USA) or indocarbocyanine (Cy3; 1:800; or Cy5; 1:200; Millipore, Billerica, USA) coupled secondary antibodies were used. The first and secondary antibodies were diluted in 1-fold Roti-Immu-no-Block without Triton X-100 (Carl Roth, Karlsruhe, Germany). For visualization of cell nuclei cells were co-stained with DAPI (Invitrogen, Karlsruhe, Germany). For negative controls, primary antibodies were omitted in each experiment.
was added for the solubilization of formed crystals. We seeded the same amount of cells at day 0 and performed an MTT-Assay on days 3, 5, and 7. At every time point control cells and cells treated with human recombinant IFNγ were tested. We used the same dose of human recombinant IFNγ, which was used in the murine study (1000 U/ml).

REAL-TIME QUANTITATIVE PCR
RNAs from triplicate each. Every experiment in IFNγ (PBS-treated) were analyzed in at least 3 independent cultures in triplicate each. The resulting data sets were

STATISTICAL ANALYSES
Experiments were repeated with independent cultures at least three times in triplicate each. The resulting data sets were statistically analyzed and illustrated using the GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA, 2003) software. For approval of statistical significance between groups, a two-tailed unpaired t-test was performed. P-values <0.05 were considered to indicate significant differences.

CSF COLLECTION AND IFNγ ELISA
Lumbar CSF was obtained from patients admitted to our department for diagnostic purposes. Patients suffered from multiple sclerosis (three samples), peripheral nervous plexus affections (one samples), Alzheimer’s disease (one sample), viral myelitis (one sample), or stroke (one sample), were compared to control CSF (three samples). All CSF specimens were immediately centrifuged, aliquoted and stored at −35°C. Ethics approval for the use of human CSF was obtained from the institutional ethics committee. For IFNγ detection in the CSF samples an IFNγ ELISA by R&D systems (R&D systems, Wiesbaden-Nordenstadt, Germany) was used. The experimental protocol followed the manufacturer’s instructions. For statistical testing 3 control patients were compared with 3 MS patients with an unpaired two-tailed t-test.

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