Investigation of the Therapeutic Potential of (Stem) Cell Containing Human Umbilical Cord Blood Fractions for Repair of Ischemic Neuronal Damage
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Doreen Melanie Reich

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Abstract

This dissertation includes two publications and one chapter of so far unpublished material investigating the neuroprotective potential and mechanisms of umbilical cord blood and stem cell fractions thereof. In direct and indirect co-cultures of cord blood cells under investigation with hypoxically damaged neuronal cells or oxygen and glucose deprived rat hippocampal slice cultures apoptosis, necrosis, soluble mediators, and adhesion molecules were analyzed.
Investigation of the Therapeutic Potential of (Stem) Cell Containing Human Umbilical Cord Blood Fractions for Repair of Ischemic Neuronal Damage

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2 Introduction

2.1 The mononuclear fraction of human umbilical cord blood as a source of adult stem and progenitor cells for cell based regenerative therapies

In laboratories all over the world scientists have been working on the characterization and utilization of stem and progenitor cells for regenerative cell-based therapies of oncologic, immunologic and degenerative diseases. The term “stem cells” defines a special type of cells that are distinguished from other kind of cells by mainly three important characteristics: i) they are immature, not specialized cells ii) they have the ability to renew themselves indefinitely through numerous cell divisions while maintaining the undifferentiated state and iii) they are capable of becoming tissue- or organ- specific cells, highly specialized in function and structure.

In mammals there are three types of stem cells: germinal, embryonic, and somatic stem cells, whereas the latter are also known as adult, foetal or tissue- derived stem cells. Those three major types of stem cells are characterized by different degrees of lineage potential varying from the totipotency of zygotes to the pluripotency of embryonic cells and to the multipotentiality of adult stem cells.

For the purpose of regenerative cell therapies embryonic and adult stem cells are of special interest. Embryonic stem cells originating from the inner cell mass of the blastocyst, proliferate by symmetric cell division. Other than adult stem cells embryonic stem cells can be kept in in vitro cultures for more than a year without differentiation and are than referred as embryonic stem cell lines (Pal et al., 2009; Irion et al., 2009). In general stem cells only rarely divide. Division needs appropriate external stimuli given by chemical and physical signals in the microenvironment of the cells.

In some adult tissues, such as bone marrow, muscle and brain discrete numbers of adult stem cells reside in so called stem cell niches. Their primary role is to ensure maintenance of tissue characteristics by generation of replacement for tissue that got lost in the normal tissue turn over, by injury or by disease (Serakinci et al., 2006).

Research on adult stem cells began in the 1950s when it was discovered that bone marrow contains two types of stem cells; mesenchymal- and hematopoietic stem cells. Hematopoietic stem cell populations which are also found in human umbilical
cord blood (HUCB) can give rise to all types of blood cells and also non-hematopoietic neural, cardiac, epithelial, hepatocytic, and dermal tissue (van de Ven et al., 2007; Rogers et al., 2007, Jay et al., 2004). Given the unique potentials the adult stem and progenitor cells of HUCB they are a remarkable alternative to the controversial use of embryonic stem cells since those are obtained from fertilized eggs of four to five days old embryos, although those eggs have been given to the research as a donation.

HUCB stem cells can be obtained non-invasively from the umbilical cord, a waste product after birth. Therefore, there are no ethical concerns and no legal restrictions comparable to the concerns of isolation and utilization of embryonic stem cells. In addition, there is no known risk of developing malignancy. Also when compared to other sources of adult stem cells and progenitor cells HUCB and HUCB stem cells offer some remarkable advantages: they are proven to have a low virus contamination (Frey et al., 2009), strong homing capabilities (Rosenkranz et al., 2010) and long term storability (Sullivan et al., 2008). The low risk of a graft versus host reaction due to immature T cells producing only low amounts of activating cytokines (Rioradan et al., 2007) and a low amount of natural killer cells in the lymphoid fraction of umbilical cord blood (Garderet et al., 1998) adds to the advantages of the use of umbilical cord blood cells. The successful and safe utilization of the potential HUCB stem cells has been impressively shown in about 15,000 cord blood transplantations in hematopoietic, cardiovascular and neurologic diseases performed since 1988 (Frey et al., 2009).

### 2.2 The neural plasticity of adult progenitor and stem cells of human umbilical cord blood

Human umbilical cord blood is rich in adult stem and progenitor cells, capable to differentiate in hematopoietic cell lines. Hematopoietic progenitor cells contain colony forming units (CFU) for granulocytes and macrophages (CFU-GM), erythroide cells (BFU-E), granulocyte-erythroide-macrophage-megacaryocyte (CFU-GEMM) as well as myeloid-lymphoid initiating cells (LTC-ICs) and therefore are the origins of all blood cell lines (Broxmeyer et al., 2006). According to the standard protocol of the International Society of Hematotherapy and Graft Engineering (ISHAGE) hematopoietic human umbilical cord blood cells represent one percent of the mononuclear cell fraction (MNC) of HUCB. In 1995 the ISHAGE Stem Cell
Enumeration Committee characterised stem cells by the expression of the surface markers CD (cluster of differentiation) 133/CD45 and CD34/CD45 as well as by a specific size and granularity shown by a characteristic deflection of the laser beam in the flow cytometer (Sutherland et al., 1996). Depending on the frequency of those markers a distinction is made between CD133+/CD34-, CD133+/CD34+ and CD133-/CD34+ phenotypes which are characterised by decreasing pluripotency (Yin et al.; 1997 and Pranke et al., 2001). In addition to mesodermal hematopoiesis it has been shown that HUCB stem cells can give rise to non-hematopoietic ectodermal tissue by crossing lineage borders (Seidel et al., 2003).

For regenerative medical therapies the mononuclear fraction of human umbilical cord blood (HUCB-MNC) as well as HUCB stem cells are of major interest, since both fractions can be differentiated into cells showing neuronal characteristics. MNC were shown to express the neuronal markers Musashi-1 and TUJ-1 and the astrocytic marker glial fibrillary acidic protein (GFAP) and there is transcription of nestin under the influence of retinoic acid and neuronal growth factor (Sanchez-Ramos et al., 2001). Cultivated with beta-mercaptoethanol HUCB-MNC develop a neuronal phenotype characterized by the expression of the markers NeuN, neurofilament and GFAP as well as by the transcription of the MAP2 and the nestin gene (Ha et el., 2001).

If retinoic acid, a vitamin D derivate being a significant factor in the neuronal ontogenesis of vertebrates, is added to CD 133+/CD45+ cells, cultures express the neuronal markers beta III tubulin, neuronal specific enolase, neurofilament M and H, microtubuli assoziated protein 2, neuronal nuclei (NeuN), astrocytic marker (GFAP), and oligodendrozytic markers (myelin-basic protein [MBP], cyclic nucleotid phosphodiesterase [CNPase]) (Jang et al., 2004; Baal et al., 2004). The CD34+/CD45+ stem cell fraction also showed markers of early neurogenesis like neuronal nestin, MAP 2, beta III tubulin and astrocytic GFAP, and, oligodentrcytic galactocerebroside (Gal-C) in presence of retinoic acid and the neurotropin brain-derived neurotrophic factor (BDNF) (Buzanska et al., 2002).

That linage crossing differentiation into cells showing ectodermal characteristics makes HUCB-MNC and HUCBC highly promising tools for the treatment of central nervous system diseases. This is particularly valuable for neuronal disorders accompanied with cell loss since there is currently only symptomatic treatment but no cure available.
2.3 The ischemic stroke

The brain is one of the most central organs of the body. It controls and coordinates fundamental life keeping functions and regulates the complex behavior of human beings. Malfunction of the brain can cause very severe diseases and lasting disabilities. Stroke, a medical emergency, is caused by critical decrease in blood supply of the brain. Depending on whether there is an obstruction or a disruption of a blood vessel leading to an acute circulatory disturbance or to bleeding in the brain, a distinction is drawn between an ischemic stroke and a hemorrhagic stroke occurring in 85% or 10-15%, respectively. Causes for an ischemic stroke include thromboembolic, microangiophatic and hemodynamic mechanisms. After cardiovascular diseases and cancer, stroke is the third leading cause of death and the most common reason for disabilities in adulthood (Kolominsky-Rabas et al., 2006). Stroke is the most common acute disease of the brain and a reason for degradation of quality of life in adulthood in industrialized countries (Mäkinen et al., 2006 and European Stroke Organisation). For e.g. in the US every 40 seconds a person sustains a brain stroke which accumulates to about 795,000 new or reoccurring strokes per year (Lloyd-Jones et al., 2008) (Heart Disease and Stroke Statistics 2009 Update Circulation. 2009 Jan 27;119(3):e21-181. Epub 2008 Dec 15). In Germany too brain stroke is one of the most commonly occurring severe diseases. As per the last available statistics since 2006, with 65,133 incidents, stroke ranks at third position.

To maintain the functional- and structural metabolism of brain tissue an optimal supply of oxygen and glucose is crucial since regular brain function is dependent on degradation of glucose. Oxygen being essential for the strictly aerobic degradation of glucose is permanently delivered by blood flow. Under normal conditions the brain, not having the ability to store oxygen and glucose, withdraws 30-40% of oxygen and 2-5% of glucose from the blood. The consequences of constrained blood supplies depend on the degree of the insufficiency in blood supply and the duration of ischemia. Data from experimental total ischemia show that as early as a few seconds after a blood supply reduction to 50 percent of the normal average cerebral blood flow (CRF, 0.8ml/g/min, in humans) clinical symptoms occur. No free oxygen can be measured after two to eight seconds of anoxia. Loss of consciousness appears after 12 seconds and after 30 seconds no
electric activity is measurable in the electroencephalogram. If anoxia continues for three to four minutes it results in an irreversible damage of the brain tissue. Finally, nine minutes of anoxia cannot be survived except under hypothermic conditions (Hartmann, Der Schlaganfall, 2001). In 1993 Gomez epitomized the sensitivity of brain tissue against reduced oxygen partial pressure in the sentence “Time is brain” (Gomez, 1993). His assertion was impressively underlined by a meta analysis investigating the average time of the development and the size of an average stroke. According to this study an average stroke with a size of 54 millimeters (19-100 ml) leads within an average time of ten hours (6-18 hours) to the death of 1.9 millions neurons and 14 billions synapses and to a destruction of twelve kilometers of myelinated nerve fibers per minute (Eschenfelder et al., 2006; Saver JL, 2006).

The severity of insufficient circulation is also a crucial factor for the development of the damage. Based on early findings made by Opitz et al. describing a sequential correlation between the severity of a hypoxia and energy consuming processes in the brain, Symon et al. postulated a concept of two viability thresholds of ischemia (Opitz et al.,1950; Symon et al.,1977). The essence of the work is the definition of two critical CBF thresholds at which the electrical and structural integrity of the cell membrane can not be maintained anymore (also see paragraph 2.4). This concept also describes that the more complex functions of the brain are the more sensitive in their tolerance against reduction of oxygen. In addition to this correlation to complexity a cell type relation is included. It has been shown that CA-1 neurons of the hippocampus exhibit a strong sensitivity against oxygen deficiency. Neurons forming the basal ganglions and neurons of the cortex are very sensitive as well while glial cells and the blood vessel endothelium tolerate reduced oxygen supply best (Banasiak et al., 2000).
2.4 The relationship between the severity of reduction in oxygen supply and neuronal cell death and the concept of a penumbra

The above mentioned concept of two viability thresholds defines two critical blood flow rates impairing function of neuronal cells as electrical or membrane failure. Those blood flow thresholds define lower and upper CBF limits beyond which brain tissue is destroyed or not harmed. Beyond the lower threshold, at a blood circulation of <0.1ml/g/min content of tissue oxygen and glucose tend towards zero (Endres and Dirnagl, 2002; Kaufmann et al., 1999).

The concomitant lack of substrates switches the cell metabolism to anaerobic glycolysis leading to an energy crises through high consumption of glucose by generating a very reduced amount of ATP. Lactic acid produced in this metabolic state increases the permeability of the cell membrane and leads to an intracellular accumulation of potassium and calcium concentrations. Resulting anoxic depolarization of the cell membranes leads to collapse of intra- and extra cellular ion gradients accompanied by release of excitatory and inhibitory amino acids e.g. glutamate. By activation of glutamate-dependent calcium channels those high concentrations of glutamate support the influx of calcium. Calcium toxicity activates enzymes of osmolysis, lypolysis and proteolyses irreversibly leading to the death of cells, called necrosis (Siesjo, 1992). Necrotic intracellular processes include collapsing of the respiratory chain of mitochondria and therefore loss of cellular energy and lysis of the cell. Those lytic processes inevitably lead to the loss of the cell membrane and therefore to the release of disintegrated intracellular proteins and nucleoid acids into the intracellular space (Endres et al., 1997; Eliasson et al., 1997; Ferrer, 2006). That inflammatory milieu activates microglia and macrophages to phagocyte the necrotic tissue leaving cystic degenerated brain tissue. The region where necrosis happens is called the necrotic core of the infarction.

Intercalated between the necrotic core and the not impaired regularly supplied brain tissue lies a rim of tissue that, according to the bright areas of the moon during a total eclipse, was named penumbra and since then has been a standard term of the medical language (Astrup et al., 1981). The ischemic penumbra is characterized by significant CBF threshold of 0.2 to 0.4 ml/g/min (Endres and Dirnagl, 2002; Kaufmann et al., 1999). Characteristic for that level of hypoperfusion neurons are not being able to maintain electrical activity but are able to preserve function of their ion channels and structural integrity (Ferrer, 2006). However, without restoration of blood flow the
singular status of the penumbra can not be maintained for more then 6-12 hours. This is attributable to an expansion of the ischemic core consuming the penumbra tissue. This is due to repeated peri-infarct depolarisations caused by glutamate massively released from the ischemic core.

The extensive surplus of glutamate leads to an over stimulation of ionotrophic glutamate receptors and therefore to massive influx of calcium ions inhibiting generation of ATP in the mitochondria. In the area of tension caused by hyperexcitation and simultaneous reduced supply of ATP apoptotic cascades are activated (Ferrer, 2006). Without restoration of oxygen supply apoptotic signal cascades will consequently lead to degradation of nucleic acids and therefore to unavoidable death of the cells within the penumbra. However, the reversible character of those cascades also allows interruption of the signal transduction making the penumbra tissue a unique target to medical interventions aiming to preserve as much brain tissue as possible.
2.5 The acute treatment of the ischemic stroke
Since autumn 2004, recombinant tissue plasminogen activator (r-tPA) has been the approved gold standard for the acute treatment of ischemic stroke. However, the application of r-tPA is strictly limited to highly specialized intensive care wards, so called stroke units. Thrombolyzation by r-tPA allows a rapid revascularisation and is therefore strictly limited only for patients suffering from embolic strokes confirmed by exclusion of intracranial bleeding using imaging techniques (Klijn et al., 2003). R-tPA treatment is also clearly limited through a narrow time window: It is only approved for use within three hours of onset of the first stroke symptoms (Toni et al., 2005) and optimal results can be achieved if given within 90 minutes after stroke onset (Hacke et al., 2004). In fact, due to this narrow time window and due to contradictions in the use of r-tPA, this treatment is only available to circa 5% of stroke patients and a treatment beyond the time window involves high risk of severe brain hemorrhages since the damage of the basal membrane of the endothelia increases with the duration of the ischemic condition (Albers et al., 2004).
Therefore, the challenge for experimental stroke research is to find supplementary and less time-dependent therapeutic strategies to the lysisation treatment.

2.6 Neuroprotection as a concept to limit neurodegradation and cellular damage after ischemic stroke
Beyond acute phase treatment approaches to improve the CBF neuroprotection is in the focus of stroke therapy. The term neuroprotection describes every strategy or a compilation of strategies aiming to antagonize, to interrupt or to slow down destructive processes within the nervous system that would cause irreversible damage (Ginsberg et al., 2008). Starting in the late 1990s neuroprotection in stroke therapy has gained more and more importance. This is impressively illustrated by many scientific publications shown in the NCBI database. Beginning in the year 1990, 2,314 articles dealing with neuroprotection and stroke/ischemia are listed. Beyond tests in animals, progress in the field of neuroprotection can be seen from the number of performed clinical trials. In November 2009 there were 160 trials listed in the Stroke Trial Registry of Center of Washington University School of Medicine. During the rapidly advancing progress in science in the last decade many potential strategies have been suggested that could positively influence the pathophysiological pathways of acute ischemia. Neuroprotection in this sense stands for interruption of
the pathological signal cascades of calcium and glutamate toxicity. In this context the scientific research focuses on the single components within a class of substances including calcium and sodium blockers, calcium chelating agents, GABA-, AMPA- and NMDA antagonists, nitrogen dioxides and inflammation inhibitors. However, given the complexity of events occurring during cerebral ischemia it might be elusive that single agent trials will demonstrate path-breaking benefits. In the future combinational therapies might gain more scientific attention.

2.7 General considerations of the utilization of adult stem cells in the treatment of ischemic stroke

In general adult stem cell therapy for stroke can be divided in an endogenous and an exogenous path.

The ultimate goal of both approaches is to improve functional recovery not only by replacement of the infarced tissue in an organotypic manner but also by reestablishment of a functional neuronal circuitry supported by a working glial network.

The endogenous approach has become an option since the biological dogma that regeneration of brain tissue is not possible has been unequivocally proven wrong with the discovery of neurogenic regions in the adult brains of many different mammalian species. In fact, neurogenesis occurs in the subventricular zone of the lateral ventricle and in a layer between the hilus and the granule cell layer of the hippocampal dentate gyrus in human. Additionally, some studies also indicate the existence of neurogenesis in the striatum, spinal cord, and neocortex in the adult rodent brain (Palmer et al., 1999; Yamamoto et al., 2001; Palmer et al., 1995).

The discovery that the adult brain is a place of neurogenesis raises the opportunity that new born neuronal cells may have the potential to restore neurons and glia in brain areas damaged by stroke. In response to ischemic injury there is even a special enhancement of neurogenesis. Following global ischemia a more than 10-fold amplification of neurogenesis in the hippocampal dentate gyrus and in the subventricular zone of the lateral ventricle has been observed up to two weeks, although this neurogenesis did not lead to regeneration of pyramidal cells that are damaged in global ischemia (Liu et al., 1998; Tonchev et al., 2005). Likewise there is a neurogenic response to focal ischemic injury with a peak in proliferation at around one to two weeks (Arvidson et al., 2002; Parent at al., 2002). However, to date there
has been no convincing evidence that new born cells from the hippocampal dentate gyrus ever migrate out to replace lost cells after ischemia. By contrast, neuroblasts originating from the subventricular zone of the lateral ventricle have been shown to divert from their normal migration route toward the olfactory bulb but instead migrate toward the ischemic penumbra (Arvidson et al., 2002; Parent et al., 2002; Jin et al., 2003b). However, although some migrated cells developed makers of matured neuronal cells, in none of those studies a full maturation, long term survival or synaptic integration into the cortical neural circuitry could be observed.

Apparently, endogenic stroke-induced baseline neurogenesis is, also after pharmacological stimulation, not sufficient to functional replace lost tissue and might need external augmentation to provide a meaningful regeneration. Therefore, focus of scientific attention has shifted to transplantation of cells of various cell types. For this approach neuronal stem cells, embryonic stem cells as well as bone marrow and cord blood-derived stem cells have been employed.

With the external approach, that not only makes use of the differentiation capacity but also of migratory potential of stem cells, come some remarkable advantages: control over cell numbers, cells can be pre-differentiated into the desired cells types, cells can be administered locally, if applicable and fate of transplanted cells is easier to trace since cells can be pre-labeled before administration. But most of all, many findings promise an expansion of the narrow therapeutic time window using external cell therapies. Bone marrow cells have even been administered a full week after focal ischemia and even if the observed benefit might not result from graft-derived tissue it argues for other mechanisms then cellular replacement of damaged tissue.

In the resent years several possible modes of action of transplanted adult stem cells have come under intensive discussion:

i) Systemically administered stem cells might cross the blood brain barrier, migrate into the degenerated region and restore the impaired tissue by transdifferentiation (Chu et al., 2004, Taguchi et al., 2004) ii) Stem cells might provide trophic factors enhance endogenous neurogenesis and stimulate restoration of damaged neuronal networks (Chu et al., 2004, Taguchi et al., 2004) iii) Administered stem cells might affect micro environmental conditions e.g. by suppression of proinflammatory cytokines like TNFα, IL-1ß and IL-2 and therefore enhance conditions for newly generated endogenous neuronal cells (Vendrame et al., 2005).
2.8 Specific neuroprotection mediated by stem cells of the umbilical cord blood in animal models of the ischemic stroke

In the broadest sense the term neuroprotection also describes recovering of neurological functionality based on the ability of stem cells to differentiate across tissue lineage boundaries. Especially, due to limited endogenous self-repair capacity of the brain, cell-based therapies are a highly promising option for the therapeutic intervention of stroke and stem cells from HUCB promise a great potential. Based on experimental knowledge on biochemical and molecular processes of apoptosis gained during the last decades the tissue of the penumbra is considered as a valuable target for neuroprotective cellular therapies.

First indications for a clear reduction of the neuronal loss of function throughout transplantation of the mononuclear cell fraction of HUBC have been found in animal models of ischemic stroke. The intention to establish animal models is to mimic diseases in complex organisms to study pathophysiological reactions and therapeutic modes of actions. To create an experimental ischemic stroke that mimics the situation in humans a transient or permanent occlusion of the arteria cerebri media (MCAO) is performed in various species, predominantly rodents. That way the infarced areas occur in the cerebral cortex and in the striatum. Using the MCAO model in their pioneering work, Chen et al. were non-recurrently able to show the expression of neuronal (NeuN and MAP-2) and astroglial markers (GFAP) on intravenously transplanted HUCB-MNC (Chen et al., 2001). In 2004 Vendrame et al. showed a dose-dependent relationship between the amount of intravenously administered HUCB-MNC and the reduction of infarct volume and improvement of behavioral deficits in a rat MCAO model (Vendrame et al., 2004 and Willing et al., 2003a/b). According to their results a significant reduction of the infarct size was reached at doses of 10E+07 HUCB-MNC. Behavioral performance reached significant levels after the administration of 10E+06 HUCB-MNC. Based on those results Newman et al. showed a targeted migration of transplanted HUCB-MNC towards the damaged brain tissue (Newman et al., 2006). Additional to the dose dependency a temporal relation was reported by Newcomb et al., 2006. They were able to show significant improvements in behavior and a diminished poststroke inflammation due to reduced infiltration of granulocytes and monocytes and to reduced activation of microglia at 48 hours following MCAO (Newcomb et al., 2006). On the basis of proven plasticity of HUCB-MNC in vitro it was originally assumed that the observed improvement seen in animal models after cell transplantation was due...
to a functional recovery of the damaged tissue through cellular replacement by differentiated donor cells. However, transdifferentiated donor cells were transient and too minor to cause significant improvements in motor functions. A path breaking study by Borlongan and co-workers gave a hint that observed impressive improvements after HUCB-MNC transplantation might not only be due to transdifferentiation of the grafts. They showed that entry of grafted cells into the brain is not a prerequisite for acute neuroprotection since therapeutic molecules secreted by HUCB-MNC have the ability to cross the blood brain barrier (Borlongan et al., 2004). The molecular signalling of those therapeutic molecules is assumed to include cascades of chemokines, cytokines and integrines and the activation of adhesion molecules, though the mode of action in regard to recuing cells within the penumbra remains elusive.

Since the MNC-HUCB fraction is very heterogeneous it seems to be reasonable to investigate the capacity of purified or selected stem and progenitor cells. Especially since there is a spontaneous increase in the CD45+/CD34+ cell count in the peripheral blood after acute cerebral ischemia in humans the investigation of this cell type is highly interesting. It could be considered as an insufficient self-repair attempt of the body and therefore it seems plausible investigate whether a transplantation of CD34+ cells is beneficial after stroke.

So far there is only one published study that investigated the impact of pure HUCB stem or progenitor cells on MCAO animal models of stroke: Application of HUCB-CD34+ stem cell fraction on immunocompromised MCAO mice showed infract size reduction associated with enhanced neurovascularization and stimulated endogenous neurogenesis (Taguchi et al., 2004).
2.9 Objectives

What we know about stroke-related pathophysiological pathways today has been mostly gained from experimental research utilizing rat or mice animal models of focal ischemia. That data is of inconsiderable value to understand the impact of stroke not only on the brain but also on other organ systems. Complexity of metabolic, electric, and immunologic stroke-induced cascades and the speed of damage development contribute to the problem to understand and to treat stroke in a sufficient manner. The cell transplantation approach builds upon the complexity of stroke events since the transplant is very heterogeneous and complex in itself. The cell culture models used in this study therefore focus on the interplay of transplanted HUCB cells and hypoxically damaged neuronal cells in reduced but still sufficiently complex experimental set ups.

Using those in vitro approaches this study addresses the following questions:

i) Is administration of enriched stem cell fractions obtained from HUCB more beneficial in reduction of levels of apoptosis and necrosis as compared to application of unfractioned HUCB-MNC?

To know whether there are stem cell specific anti-apoptotic and/or anti-necrotic mechanisms would be groundbreaking for considerations in future clinical applications since stem cells are rare and enrichment is an elaborate procedure. If it should turn out that pure stem cell fractions are not highly superior to unfractioned HUCB-MNC in the treatment of neuronal injury, provision of sufficient cell numbers would be less problematic. In this study the neuroprotectional potential of unfractioned HUCB-MNC was compared to anti-apoptotic and anti-necrotic potential of stem cell containing and stem cell depleted fractions of HUCB. Enrichments and depletions of stem cells were achieved by magnetic cell separation methods that resulted in highly purified cell fractions. Both stem cell enriched and depleted fractions of HUCB were co-cultured with hypoxically damaged neuronal cells representing a self-developed cell culture model of neuronal hypoxia based on fully differentiated SH-SY5Y cells.
ii) Is direct application of HUCB-MNC superior to indirect application of HUCB-MNC in regard to levels of neuronal apoptosis and necrosis?

Comparison of direct and indirect application modes allows further insights into conceivable modes of action of transplanted cells. This approach is very useful to examine whether cell-derived survival signals for neuronal cells are mediated by cell-cell contacts or as soluble factors. Therefore, intercellular interactions like the expression of adhesion molecules were scrutinized along with release of a broad range of chemokines and cytokines. Indirect application was performed using cell impassable cell culture inserts separating administered HUCB cell fractions from hypoxically injured neuronal cells.

iii) To what extent are results gained from the utilized cell culture model of hypoxically induced neuronal damage applicable to damage profiles of complex nervous tissue?

The self-developed cell culture model of hypoxically damaged neuronal cells has proved to be very useful to investigate interactions between transplanted cell fractions and injured neuronal cells. The present study investigates whether findings made in this model apply to hypoxically injured organotypic rat hippocampal slices, which comprise all neural cell types in a complex three-dimensional network. Focus on alterations in necrotic cell death and in migration behavior of cellular fractions within HUCB-MNC.

The following chapters include publications that examine and discuss the above mentioned questions in detail.
3 Preface to Chapter 1

Utilization of animal models of stroke are highly suitable to investigate effects of cell transplantation on complex parameter like sensorimotor and cognitive functions and histological outcome of the subjected animals. However, to learn more about the therapeutically capacity and underlying mechanisms reduction of complexity seems to be reasonable. The following chapter introduces a human in vitro model of neuronal hypoxia consisting of fully differentiated vulnerable SH-SY5Y cells undergoing apoptosis and necrosis in the post-hypoxic state. The progression of apoptosis and necrosis was investigated after HUCB had been added. Special respect was drawn to graft secreted (soluble) factors and expression of adhesion molecules on target cells.
4 Evidence for Neuroprotective Properties of Human Umbilical Cord Blood Cells After Neuronal Hypoxia in vitro
4.1 Abstract

Background: One of the most promising options for treatment of stroke using adult stem cells are human umbilical cord blood (HUCB) cells that were already approved for therapeutic efficacy in vivo. However, complexity of animal models has thus far limited the understanding of beneficial cellular mechanisms. To address the influence of HUCB cells on neuronal tissue after stroke we established and employed a human in vitro model of neuronal hypoxia using fully differentiated vulnerable SH-SY5Y cells. These cells were incubated under an oxygen-reduced atmosphere (O2 < 1%) for 48 hours. Subsequently, HUCB mononuclear cells (MNC) were added to post-hypoxic neuronal cultures. These cultures were characterized regarding to the development of apoptosis and necrosis over three days. Based on this we investigated the therapeutic influence of HUCB MNC on the progression of apoptotic cell death. The impact of HUCB cells and hypoxia on secretion of neuroprotective and inflammatory cytokines, chemokines and expression of adhesion molecules was proved.

Results: Hypoxic cultivation of neurons initially induced a rate of 26% ± 13% of apoptosis. Hypoxia also caused an enhanced expression of Caspase-3 and cleaved poly(ADP-ribose)polymerase (PARP). Necrosis was only detected in low amounts. Within the next three days rate of apoptosis in untreated hypoxic cultures cumulated to 85% ± 11% (p ≤ 0.001). Specific cytokine (VEGF) patterns also suggest anti-apoptotic strategies of neuronal cells. Remarkably, the administration of MNC showed a noticeable reduction of apoptosis rates to levels of normoxic control cultures (7% ± 3%; p ≤ 0.001). In parallel, clustering of administered MNC next to axons and somata of neuronal cells was observed. Furthermore, MNC caused a pronounced increase of chemokines (CCL5; CCL3 and CXCL10).

Conclusions: We established an in vitro model of neuronal hypoxia that affords the possibility to investigate both, apoptotic neuronal cell death and neuroprotective therapies. Here we employed the therapeutic model to study neuroprotective properties of HUCB cells. We hypothesize that the neuroprotective effect of MNC was due to anti-apoptotic mechanisms related to direct cell-cell contacts with injured neuronal cells and distinct changes in neuroprotective, inflammatory cytokines as well as to the upregulation of chemokines within the co-cultures.
4.2 Background

Acute ischemic stroke is characterised by the immediate depletion of oxygen and glucose in brain tissue. A residual cerebral blood flow (CBF) of $\leq 6 \text{ cm}^3 \times 100\text{g}^{-1} \times \text{min}^{-1}$ representing severe ischemia is associated with a nearly total loss of energy on vulnerable neurons. Ischemia therefore rapidly culminates in the formation of a necrotic core [Kaufmann et al., 1999]. In the penumbra, mild ischemia (CBF 11-20 $\text{cm}^3 \times 100\text{g}^{-1} \times \text{min}^{-1}$) leads to the activation of complex neurochemical cascades of cell death, mainly apoptosis. In principle these apoptotic cascades are reversible and form an important aspect of the penumbra concept, which is the major target of therapeutic interventions [Kato et al., 1999; Saito et al., 2005]. Recent findings indicate that transplantation of external cell fractions could accompany established therapeutic procedures limited by narrow time windows [Newcomb et al., 2006], but the underlying processes are still rather unclear.

Our insights into pathophysiological processes and new therapeutic strategies have mostly been obtained from animal models of focal cerebral ischemia [Ahmed et al., 2000; Mergenthaler et al., 2004] and rodent organotypic hippocampal slice cultures [Lewczuk et al., 2000; Laskowski et al., 2005; Choi et al., 2007]. However, the complexity of those systems has limited the detailed understanding of mechanisms related to ischemic brain injury [Traystman et al., 2003] and possible interfering effects of cellular therapies [Haas et al., 2005] so far. Furthermore, results obtained from rodent models are not completely and unobjectionably transferable to human therapy [Heiss, 2002; Heiss et al., 2003]. Consequently, experimental expenditure and ethical considerations demand in vitro models representing the main properties of stroke-related processes as neuronal apoptosis to accompany more complex model systems. This would allow answering explicit questions concerning the role of cell-cell interactions and production of metabolites to verify observations made in in vivo models. It furthermore offers the possibility to precisely manipulate extracellular environments.

Well described human neuronal cell lines exhibit a multitude of characteristics of typical central-nervous-system (CNS) neurons, overall cell material can be achieved in large quantities. Therefore, human neuronal cell lines, such as the teratocarcinoma NT-2 cell line, became useful tools to study the effects of hypoxic conditions on neurons [Rootwelt et al., 1998]. However, the utilisation of NT-2 neuronal cultures is
restricted by time-consuming and expensive differentiation periods of up to 44-54 days [Paquet-Durand et al., 2003; Pleasure et al., 1992] that are also sensitive to environmental disturbances. In contrast, the SH-SY5Y neuroblastoma cell line was shown to be differentiated into neuronal cells within a comparatively short time of 16 days [Encinas et al., 2000]. Furthermore, the cell line fits major relevant criteria (high vulnerability, irreversible differentiation into pure neuronal cells) to serve as a model of hypoxic injury of central neurons [Lopez et al., 2000]. Hence, our exclusive human model of neuronal hypoxia forms the basis to identify possible anti-apoptotic neuroprotective potentials of therapeutic supplements. Mononuclear cells (MNC) from human umbilical cord blood (HUCB) were shown to improve functional outcome of animals after focal cerebral ischemia. The cellular effects causing the observed benefits are not fully understood for these cells [Chen et al., 2006; Vendrame et al., 2004]. In this context we investigated whether injured post-hypoxic neuronal cells or MNC initiated an anti-apoptotic response mediated by cytokines or chemokines.
4.3 Methods

4.3.1 Cultivation and differentiation of neuronal cells
All experiments were performed using SH-SY5Y human neuroblastoma cells (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) between passages 4-7. The common medium was Dulbecco's Modified Eagle Medium (DMEM, high glucose 4.5 g/l, L-Glutamine 580 mg/l; PAA, Pasching, Austria) with penicillin G (10,000 U/ml; PAA Laboratories, Pasching, Austria) and streptomycin (10 mg/ml; PAA Laboratories, Pasching, Austria). Cells were maintained in MM in a humidified atmosphere with 5.5% CO$_2$ at 37 °C (Table 1). When cultures achieved subconfluence, cells were subcultured with trypsin/EDTA (PAA Laboratories, Pasching, Austria). Differentiation was carried out according to the protocol of Encinas and colleagues [17] but was adapted as follows: cells were plated at an initial density of 0.9x10^4/cm$^2$ in 16-mm-diameter cavity (Greiner Bio-One, Frickenhausen, Germany) and differentiated over a period of 16 days in relevant media and cultured thereafter according to Table 1. Cultivation procedure is illustrated by Fig. 1.
Table 1 - Composition of culture media

<table>
<thead>
<tr>
<th>day of culture</th>
<th>culture condition</th>
<th>description of media</th>
<th>supplements in DMEM</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FCS (15%)</td>
</tr>
<tr>
<td>seeding</td>
<td>normoxia</td>
<td>maintenance medium (MM)</td>
<td>x</td>
</tr>
<tr>
<td>1 - 4</td>
<td>normoxia</td>
<td>basic medium (BM)</td>
<td>x</td>
</tr>
<tr>
<td>5 - 16</td>
<td>normoxia</td>
<td>differentiation medium (DM)</td>
<td>-</td>
</tr>
<tr>
<td>16 - 18</td>
<td>normoxia / hypoxia</td>
<td>differentiation medium (DM)</td>
<td>-</td>
</tr>
<tr>
<td>18 - 21</td>
<td>normoxia</td>
<td>post-hypoxic medium (PHM)</td>
<td>-</td>
</tr>
</tbody>
</table>

The experimental design demanded varying culture media. Supplements in DMEM were purchased from: fetal calf serum (FCS, PAN-Biotech, Aidenbach, Germany), all-trans-retinoic acid (RA, Sigma-Aldrich, Steinheim, Germany), recombinant brain-derived neurotrophic factor (rhBDNF; ImmunoTools, Friesoythe, Germany), human serum albumin (HSA, PAN Biotech, Aidenbach, Germany).

4.3.2 Immunocytofluorescence of neuronal markers
The primary antibodies used against neuronal epitopes were: β-tubulin III (rabbit 10 µg/ml; BD PharMingen, Heidelberg, Germany), taurin I and neurofilament (NF) H/M (rabbit 5 µg/ml and mouse 1:200; Chemicon, Hampshire, UK), neuron-specific enolase (NSE) and microtubule-associated proteins (Map) 2a/b (mouse 1:2 and mouse 5 µg/ml; Sigma-Aldrich, Munich, Germany). Fluorochrome-conjugated secondary antibodies were purchased from DAKO, Carpinteria, CA, USA (goat anti-mouse-PE [1:200] and pork anti-rabbit-FITC [1:30]). Indirect immunostaining was processed according to manufacturer’s instructions.
4.3.3 Hypoxia and post-hypoxic cultivation
Before enddifferentiated neuronal cells were exposed to hypoxia they were refreshed with DM. Hypoxic conditions were $O_2 < 1\%$ (oxygen substituted with nitrogen) and lasted 48 hours in a 37 °C tempered and humidified incubator (Binder GmbH, Tuttlingen). After hypoxia, cultures were supplied with PHM (Table 1) and were transferred back to normoxic conditions for the following three days (Fig. 1).

![Figure 1 - Schematic illustration of experimental set up](image)

Sixteen days of differentiation were followed by 48 hours cultivation under hypoxic conditions. Following hypoxia cultures were maintained under normoxic conditions for three days and are referred as post-hypoxic. Subsequently, cultures were analysed daily.

4.3.4 Quantification of cell numbers by nuclear staining
The total number of cells was measured by nuclear staining with 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen, Karlsruhe, Germany). Cells were washed and stained with 1 $\mu$g/ml (DAPI/Methanol) for 15 minutes at 37 °C. 25 microphotographs of randomised fields were taken per cavity using a Zeiss fluorescence microscope (Carl Zeiss AG, Jena, Germany) equipped with Zeiss AxioVision-Software. The number of nuclei was automatically determined by means of Zeiss AutMess (Carl Zeiss AG, Jena, Germany).
4.3.5 Cell viability assay
Apoptosis was determined via annexin-fluorescein isothiocyanate (FITC) or annexin-phycoerythrin (PE; both 1:20 reaction buffer; BD PharMingen, Heidelberg, Germany) using fluorescence microscopy. Necrosis was identified by propidium iodide (PI) staining (1 µg/ml phosphate-buffered saline [PBS]; Bender medSystems, Vienna, Austria). Both methods detect cells in a late state of apoptosis. Apoptosis and necrosis were ascertained in different cavities. Calcein-AM (40 µM/PBS; Invitrogen, Karlsruhe, Germany) was utilised for detection of living cells.

4.3.6 Quantification of apoptotic proteins
BD Cytometric Bead Array for human apoptosis (Becton Dickinson, Erembodegem, Belgium) was used for the quantitative measurement of apoptotic proteins (cleaved PARP and Caspase-3). Manufacturer’s instructions were adapted to lyse cells directly within multi-well plates. For cell lysates differentiated cells were rinsed with PBS and incubated on ice in the provided buffer for 20 minutes.

4.3.7 Determination of adhesion molecules by immunocytochemistry and cell-based fluorescence measurement
The cultures were stained with antibodies against CD56-phycoerythrin (PE; NCAM), CD171 (L1, both Becton Dickinson, Erembodegem, Belgium), CD54-PE (ICAM-1; Immunotech, Hamburg, Germany) and CD106-PE (VCAM-1; Southern Biotech, Birmingham, Alabama, USA) in order to investigate the distribution and localisation of adhesion molecules on differentiated neuronal cells. The staining protocol proceeded as follows: Medium (DM, Table 1) was removed and dishes were washed twice with PBS. The cells were incubated with primary labelled or unlabelled antibodies (1:100) for 10 minutes at 37 °C and afterwards washed in PBS. Thereafter the cells were incubated with goat anti-mouse-PE (DAKO, Hamburg, Germany) antibody for 10 minutes at 37 °C for CD171 detection. After labelling the cultures were immersed in PBS.

The spatial distribution of adhesion molecules was observed using fluorescence microscopy (Carl Zeiss AG, Jena, Germany). The density of molecule expression was measured in cell lysates. Therefore, antibody-labelled cultures were treated with 1% Triton X 100 (Ferak, Berlin, Germany)/PBS at 37 °C. Whole-cell lysates were
transferred to black 96-well-plates (Greiner Bio-One, Frickenhausen, Germany). PE-fluorescence signals were determined with a spectrophotometer (Tecn Spectrafluor Plus, Tecn Trading AG, Switzerland) at an excitation wavelength of 488 nm and an emission wavelength of 590 nm. Specific isotypes and 1% Triton X 100 served as negative controls.

4.3.8 Preparation of Human Umbilical Cord Blood (HUCB) samples

Cord blood samples were obtained anonymously in accordance to ethical prescripts immediately after delivery. HUCB samples of healthy full-term neonates were processed according experienced methods including density gradient separation using Lymphocyte Separation Medium (PAA Laboratories, Cölbe, Germany). Gained MNC fraction was stored by freezing in the gaseous phase of liquid nitrogen after the addition of FCS/8% dimethyl sulfoxide (Serumwerke Bernburg Inc., Bernburg, Germany). Prior to use cryopreserved MNC were thawed rapidly in 75U/ml Dnase/0.5M MgCl₂ (Roche Diagnostics GmbH, Mannheim, Germany/ Sigma, Germany) and washed in RPMI (PAA Laboratories, Austria). Cell suspension was stained with carboxy fluoresceindiacetate succinimidyl ester (CFSE 5µM; Molecular Probes, Inc., Eugene, OR, USA) for 10 minutes at 37 °C.

4.3.9 Co-culture of neuronal cells and MNC

Direct co-culturing of fully differentiated neuronal cells and MNC was carried out under normoxic conditions (37 °C) over a period of 3 days following 48 hours of hypoxia. A total amount of 4.5x10⁵ CFSE stained MNC were dissolved in 500µl PHM and were added to the post-hypoxic neuronal cells (0.3x10⁵/cavity). The ratio of neuronal cells to MNC was 1:15.

4.3.10 Cytokine profiling

The supernatants from normoxic cultures on Day 21 as well as from hypoxic cultures on Day 3 post-hypoxia were collected in order to characterise soluble factors produced by cultured cells. They were detected simultaneously by means of Becton Dickinson Cytometric Bead Array. Supernatants were tested for neuroprotective (Granulocyte Colony-Stimulating Factor [G-CSF], Vascular Endothelial Growth Factor [VEGF]) and inflammatory cytokines (Interleukin [IL]-1ß, IL-6, CXCL8) as well as
chemokines (CXCL10, CCL3, CCL4, CCL5, CCL2). The detection limit was 0.02 ng/ml, except for VEGF and CCL2 (0.04 ng/ml).

4.3.11 Statistical analyses of data
Except for apoptosis and necrosis rates all results have been reported as mean values ± SD. Statistical differences were analysed by the Student’s t-test or the Mann-Whitney rank sum-test. P values of ≤ 0.05 were considered statistically significant (* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001). Apoptosis and necrosis rates were logit-transformed to obtain normally distributed quantities. The effects of time, experimental setting (hypoxia), experimental run and the investigated cavity were determined univariately, and, finally multivariately using a mixed-model approach with time and experimental setting as fixed effects and cavity and experimental run as random effects. Cytokine concentrations were compared between the normoxic and the hypoxic group by means of the Mann-Whitney rank sum test. Cytokine concentrations measured in co-cultures were compared with the sum of the concentrations obtained in the post-hypoxic neuronal cultures and in the MNC monocultures via a bootstrapping algorithm. This was performed by resampling and by the addition of concentrations of cytokines measured in post-hypoxic neuronal cultures and MNC monocultures. Results were compared with the Mann-Whitney rank sum test. The mean and standard deviations of the sum of the concentrations were also determined by bootstrapping. Bootstrapping analysis was performed using the statistical software package “R” [The R Project for Statistical Computing; Ihanka et al., 1996]. Mixed-model analyses were performed using PROC MIXED from the statistical software package SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Box plots (if applicable) and univariate analyses were determined using the software package SPSS (SPSS Inc., Chicago IL, USA).
4.4 Results

4.4.1 After differentiation SH-SY5Y exhibited neuronal morphology and specific neuronal markers

Sixteen days of differentiation yielded cultures of fully matured neuronal cells shown in Fig. 2A, B. Following the seventh day of differentiation, the majority of SH-SY5Y cells were stained positive for the specific neuronal markers (β-tubulin III, taurin I, neuron-specific enolase [NSE], neurofilament [NF] H/M and microtubule-associated proteins [Map] 2a/b). At Day 16 all cells exhibited all of these markers. Time course of marker expression showing continuously increasing stages of differentiation is given in Fig. 2C. Markers showed typical localisation to cytoplasm and dendrites. The explicit majority of differentiated SH-SY5Y cells (73% ± 11%) resembled typical neuronal morphology with round phase-bright somata and long, terminal-branched dendrites forming a dense network (Fig. 2A, arrow 1). However, the neuronal culture is also characterised by a cell type (only 27% ± 11%) which shows abundant cytoplasm and a lack of axonal extensions (Fig. 2A, arrow 2). Remarkably, no differences were noted with regard to expression of neuronal markers. Furthermore, the number of this type of cells remained constant during the culture time.

Figure 2 - Phase contrast and fluorescence imaging of fully differentiated SH-SY5Y cells and the development of neuronal markers

Phase contrast image of fully matured SH-SY5Y cells at Day 16 (A) after seeding. Phase-bright neuronal cells (arrow 1) with long dendrites forming a dense neuronal network and cells with large cytoplasm and no axonal extensions (arrow 2). Immunocytofluorescence micrograph of fully differentiated SH-SY5Y cells (Day 16) shows cultures stained positive for the neuronal marker β-tubulin III (FITC, green) and nuclei (DAPI, blue) represented in B. Time course of detection of neuronal markers (β-tubulin III, taurin, NSE, NF-H/M and Map2a/b) on SH-SY5Y cells during the differentiation period at Day 0, 4, 7, 16 and 21 (C).
4.4.2 Absence of proliferation after termination of differentiation
At Day 4 of differentiation the total number of SH-SY5Y cells, counted by nuclear staining with DAPI, increased by twofold \((21.2 \pm 6.7 \times 10^3/cm^2)\) since seeding. Beginning on the seventh day of culture numbers of nuclei remained nearly stable \((18.2 \pm 2.2 \times 10^3/cm^2)\). Subsequent to hypoxia, there was no significant alteration in numbers of counted nuclei. Hence, the influence of oxygen deprivation induced no further proliferation and also no significant loss of cells in comparison to normoxic control cultures (Fig. 3). Therefore all following results should be seen in the context of the nearly constant numbers of neuronal cells under normoxic and hypoxic cultivation conditions. It is of note that the amount of stained nuclei as the equivalent of adherent cells gives no information about the physiologic status of these cells. On the whole the cultures include cells in a viable, apoptotic or necrotic state.

![Figure 3 - Progression of the number of nuclei after differentiation period and effect of hypoxia on cell count](image)

*Figure 3 - Progression of the number of nuclei after differentiation period and effect of hypoxia on cell count*

Nuclei were stained with DAPI and counted in fluorescence micrographs. Data were calculated from three independent experiments including multiple wells, each based on 25 micrographs. The white circles represent cultures that were cultivated under continuously normoxic conditions. The black circles illustrate the progression of the number of nuclei in post-hypoxic cultures. Under both conditions number of nuclei remained stable.

4.4.3 Hypoxia induced apoptosis in the majority of neuronal cells
Post-hypoxic and normoxic control cultures exhibited pronounced differences in the quantity of apoptotic cells as well as in cell morphology. Hypoxic conditions for 48
36 hours induced an initial apoptosis rate of 26% ± 13%. There was a continuous increase in the apoptotic cell fraction to 85% ± 11% within 3 days post-hypoxia as compared to control cultures (p ≤ 0.001; Fig. 4). The clearest effects of oxygen deficiency were seen three days following induction of hypoxia as shown by annexin-V staining (green fluorescence, Fig. 6A and 6B). Additionally, post-hypoxic cultures were characterised by retracted dendrites, indicating a loss of multiple cell-cell contacts. Debris and apoptotic bodies were found in most culture dish areas, evidencing late stage of apoptosis (Fig. 6A). In contrast, normoxic cultures displayed a much more reduced amount of apoptotic cells (Fig. 6B). Over the whole time, apoptosis in these control cultures remained stable at about 7% ± 3% (Fig. 4).

After hypoxia, on Day 0 and Day 1, number of late apoptotic/necrotic cells significantly increased up to 27% ± 13%. Further progression of propidium iodide (PI) positive cells in post-hypoxic cultures resulted in 23 ± 16% on Day 3 (Fig.5). By comparison, in normoxic control cultures, necrosis levels remained stable below 17% and therefore did not statistically differ from post-hypoxic cultures on this time point. This fact was corroborated by images that show no observable deviation in the amount of necrotic and late apoptotic cells, as indicated by PI staining (red fluorescence, Fig. 6C and 6D).

In general, an increased release of calcein-AM also indicated a strong decrease of neuronal cell viability which verifies the results of the apoptosis and necrosis rates (data not shown).
Figure 4 - Temporal progression of annexin-V positive cells in post-hypoxic and normoxic cultures

Results are derived from three independent experiments. For each experiment 75 pictures were taken. Consistently, annexin-V positive (apoptotic) values in post-hypoxic cultures are markedly higher compared to control cultures increasing to an average level of 85% ± 11% within three days. The number of apoptotic cells did not vary significantly in normoxic cultures.

Figure 5 - The progression of PI positive cells in post-hypoxic and normoxic SH-SY5Y cultures

Results are derived from three independent experiments, in which 75 pictures were taken for each analysis. Significant increases in the rate of PI positive cells were observed only at Day 0 (10% ± 6%) and Day 1 (27% ± 13%) compared to normoxic cultures (Day 0: 9% ± 10%; Day 1: 9% ± 6%). Note PI stains necrotic and late apoptotic cells. No significant difference of necrosis rate was detected of post-hypoxic cultures as well as in control cultures during the observation period.
Figure 6 - Long term effect of 48 hours of hypoxia on apoptosis (A-B) and necrosis (C-D)

Cultures at Day 3 after hypoxia were compared with normoxic cultures of the same age. Combined phase contrast / fluorescence micrographs of neuronal post-hypoxic cultures (A) and normoxic control cultures (B) show a conspicuous increase of apoptotic cells (annexin-V-staining, green fluorescence) and morphologic changes due to hypoxia (cell debris, retraction of dendrites). Propidium iodide (PI, red fluorescence) staining shows the influence of hypoxia (C) on the number of necrotic cells and cells in a late state of apoptosis compared to control cultures (D). In contrast to the level of apoptosis, there was no clear difference in the number necrotic cells following both culture conditions.

4.4.4 Hypoxia increased quantities of Caspase-3 and cleaved PARP immediately and secretion of VEGF in delay

Bar charts in Figure 7 show the concentrations of Caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP). In post-hypoxic cultures apoptotic proteins drastically rose after oxygen deprivation. The highest concentration of Caspase-3 was measured directly after hypoxia on Days 0 and 1 (2-fold and 5.8-fold, respectively). From Day 2 on, the level of active Caspase-3 sharply decreased. The distribution of cleaved PARP levels showed patterns similar to Caspase-3. This decline was accompanied by a secretion of VEGF on Day 3 after hypoxia (Fig. 10).
Figure 7 - Effect of 48 hours of hypoxia on the concentration of active Caspase-3 and cleaved PARP

Data are taken in a time course of three days after hypoxia and are based on pooled lysates of the totality of SH-SY5Y cells taken out of 12 individual wells. Data are expressed in units of protein (active Caspase-3) and cleaved protein (PARP) per millilitres. Hypoxia induced apoptosis specific proteins (Caspase-3, cleaved PARP) in a time-related manner.

4.4.5 Adhesion molecules (L1, NCAM and ICAM-1) were upregulated after hypoxia

Independent of cultivation conditions immunofluorescence analysis revealed that nearly all (98%) of the cells were positive for neurite cell adhesion molecule (L1; 98.3 ± 0.1%) and Neural Cell Adhesion Molecule (NCAM; 99 ± 0.8%) at Day 0. L1 and NCAM were detected on somata and multiple dendrites. Furthermore, a few of the neuronal cells also expressed Vascular Cell Adhesion Molecule (VCAM-1; 5.7%) and Intercellular Adhesion Molecule (ICAM-1; 14.6%) on their somata and dendrites. There was no change in relative numbers of marked cells and expression patterns in post-hypoxic cultures. To quantify the density of adhesion molecule expression, highly sensitive fluorescence measurement of supernatants of lysed cultures was performed. Here, comparative studies between cultures directly after 48 hours of hypoxia and normoxic control cultures showed a significant upregulation of L1, NCAM and ICAM-1 (Fig. 8). Hypoxic conditions determined a considerable increase in L1 and NCAM protein expressions of up to 189% ± 74% and 155% ± 50%, respectively.
respectively. Hypoxia also strongly upregulated the ICAM-1 expression up to 424% ± 251%. The level of expression of VCAM-1 was not altered by the hypoxic environment. The continued degradation of the dendritic networks did not allow assaying the specific expression of adhesion molecules at late stages of post-hypoxic cultures.

**Figure 8 - Expression of adhesion molecules after 48 hours of hypoxic cultivation**

Fluorescence intensities of cell lysates are shown after subtraction of specific isotype values with respect to normoxia. Data arise from six independent experiments. The dotted line indicates level of adhesion molecule expression (100%) in normoxic control cultures. Note statistically significant upregulation of L1, NCAM and ICAM-1 post-hypoxia.

4.4.6 Co-culturing with HUCB MNC strongly reduced apoptosis in post-hypoxic neuronal cell cultures

We found noticeable neuroprotective properties of HUCB MNC in the co-culture experiments. Untreated post-hypoxic mono-cultures of neuronal cells showed 85% ± 11% of apoptosis after three days, whereas in co-cultures with MNC the rate of apoptosis was stable at a level of 6.3% ± 1% (p ≤ 0.001). This is comparable to normoxic control cultures (7% ± 3%; Fig. 8A). Photographs of co-cultures with MNC revealed clear effort of MNC to localise next to somata and branches of post-hypoxic neuronal cells. In the course of clustering MNC mostly avoided areas that were not settled by neuronal cells (Fig. 9B). Furthermore, the administration of MNC showed positive influence on the conservation of neuronal networks as compared to cultures that did not receive any cell therapy (Fig. 6A and 6C).
Figure 9 - Effect of co-culturing of MNC on ratio of neuronal apoptosis and preservation of neuronal networks

Apoptosis was induced by 48 hours incubation of neuronal cells under hypoxic conditions. Afterwards 4.5x10^5 CFSE stained MNC were directly applied to neuronal cells (0.3x10^5/well). For three days co-cultures were observed under normoxia. In co-cultures with MNC rate of apoptosis was clearly reduced compared to post-hypoxic cultures (A). Combined phase contrast and fluorescence micrograph of post-hypoxic neuronal cells and MNC (green) in direct co-culture (B).

4.4.7 MNC increased CCL5, CCL3, CCL4 and CXCL10 in post-hypoxic neuronal cultures

The investigation of neuroprotective cytokines (G-CSF, VEGF) (Fig. 10A), inflammatory cytokines (IL-1β, IL-6, CXCL8) (Fig. 10B) and chemokines (CCL2, CCL5, CCL3, CCL4, CXCL10) (Fig. 10C) in supernatants of normoxic neuronal cultures revealed considerable amounts of CXCL8, CCL2 and VEGF. Remarkably, after three days under hypoxic conditions only VEGF was strongly upregulated (about threefold increase). After administration of MNC, VEGF was downregulated regarding to the total concentration measured in mono-cultures of MNC and in post-hypoxic neuronal cells. Inflammatory cytokines as IL-1β, IL-6 and CXCL8 were produced by MNC. The secretion of cytokines such as IL-1β and IL-6 was not altered in post-hypoxic co-cultures, whereas CXCL8 was suppressed. Interestingly, the majority of chemokines was clearly upregulated in co-cultures with MNC. We could show that CCL5, CCL3, CCL4 and CXCL10 were increased up to the tenfold (CCL3, CCL4), whereas the concentration CCL2 was not regulated.
Figure 10 - Cytokine profile in hypoxic injured SH-SY5Y cells and in direct co-cultures with MNC at Day 3

Cytokine concentrations were measured in supernatants via cytometric bead array. Data originate from five independent experiments and are expressed as ng/mg. "+" indicates significant differences in cytokine concentration of post-hypoxic SH-SY5Y mono-cultures compared to normoxic controls. Double bars are the sum of cytokine concentration from mono-cultures. "**" shows significant differences in cytokine concentration in co-cultures with MNC compared to the total of mono-cultures. Note different scaling.
4.5 Discussion

In this study, we introduce an experimental human in vitro model to investigate (i) the mechanisms of neuronal hypoxia and (ii) the interaction of neuronal cells with external stem cell-containing fractions as possible therapeutic tools. According to the differentiation protocol of Encinas et al. [Encinas et al., 2000] we obtained fully matured neuronal cells after 16 days. Previous, orientating experiments showed typical response of differentiated SH-SY5Y cells to N-methyl-D-aspartate (NMDA; 300 μM) application and most pronounced vulnerability after 48 hours hypoxic incubation (data not shown). These post-hypoxic neuronal cultures can be employed already 18 days after the seeding of naive cells. Therefore the time period until the model of hypoxic neuronal cells is available is very short. In comparison to other approaches that provide assimilable hypoxic models using teratocarcinoma cell lines [Pleasure et al., 1992] the generation of our model is shortened by at least 10 days [Paquet-Durand et al., 2004]. The proved expression of specific neuronal markers and the absence of any proliferation gave clear evidence of matured neuronal cells in the G0-phase of the cell cycle. This steady state allows making direct conclusions about the influence of any external manipulation because any change will most probably be due to these procedures. Hence, the model of neuronal hypoxia affords the possibility to investigate manifold questions concerning mechanisms triggered in response to hypoxia and therapeutic interventions e.g. application of HUCB MNC.

Following hypoxia, neuronal cultures displayed a correlation between morphological changes and increase of annexin-V positive cells as well as changes in adhesion molecule expression. The specific apoptosis marker annexin-V indicated that hypoxic cultivation preferentially induced apoptosis. Moreover, there was a continuous enlargement in number of apoptotic cells from initial 26% ± 13% until cultures almost completely consisted of apoptotic cells (85% ± 11%) within three days. Conforming to changes of morphology in post-hypoxic cultures, there was an enhanced release of calcein-AM also observable due to the loss of the integrity of membrane. This is typical for late stages of apoptosis as well as of necrosis. The model of neuronal hypoxia was approved by moderate rates of PI positive cells (23% ± 16% at Day 3 post-hypoxia). Therefore the overrun of 100% by the summation of annexin-V positive and PI positive cells is due to a proportion of cells in a late state of apoptosis that are positive for annexin-V and PI [Stroh et al., 2004]. We focused on apoptosis
because it is a reversible process which could be modulated by external stimulation [Ferrer, 2006; Fisher, 2006]. Moreover after stroke cell death in the penumbra is predominantly considered to be apoptosis [Ferrer et al., 2003; Stefanis et al., 2005; Nishioka et al., 2006; Hossmann et al., 2006]. Therefore apoptosis is a therapeutic target of anti-apoptotic therapies like stem cells or cytokines [Schneider et al., 2006; Chang et al., 2007; Shyu et al., 2006; Chairns et al., 2003].

Apoptosis in the penumbra takes place in a time span of about 72 hours after vessel occlusion in the rat after middle cerebral artery occlusion (MCAO) [Xu et al., 2006]. This time course is represented by the model system introduced in this study. The fact that there was no major alteration in the number in DAPI-positive nuclei underlines that the influence of hypoxia did not lead to an immediate destruction of the cells but allows investigating apoptosis and subsequent therapeutic interventions within 72 hours. These facts were also confirmed by characteristic changes in the morphology of post-hypoxic cells. There was a total retraction of dendrites and a resulting destruction of neuronal networks, grained cell surfaces and extended cell degradation. This damage was accompanied by an upregulation of neuronal adhesion molecules. We hypothesize that this upregulation indicates a cellular answer to compensate for a loss of direct cell-cell interaction as a consequence of the preceding hypoxic stress as described by several authors [Aubert et al., 1995, Holm et al., 1995; Martini et al., 1988]. However, the final loss of intercellular networks could not be compensated by the increased expression of neuronal adhesion molecules alone.

Neuronal cell death after hypoxia is caused by membrane depolarisation subsequent to energy failure [Hou et al., 2002; Smith et al., 2004]. The resulting calcium overload is the initial point of the activation of manifold biochemical pathways that affect caspases, free radicals and gene expression [Valencia et al., 2006; White et al., 2000]. The specific apoptotic proteins Caspase-3 and cleaved PARP were quantified to prove whether our microscopic observations are corroborated by biochemical pathways [Prunell et al., 2005; Zheng et al., 2003; Ferrer et al., 2003]. After 24 hours post-hypoxia, both marker proteins extensively increased. This was followed by a sharp drop in their expression level. This reduced amount of cleaved PARP might be due to an aggravating energy failure in the cell. This in turn is attributed to the high levels of cleaved PARP, evidenced above, which caused adenosintriphosphate (ATP) depletion [Sugawara et al., 2004; Endres et al., 1997] and which in this context
prevents the enzymatic activity of caspases. Furthermore, an increase of the expression of VEGF, a key mediator of angiogenesis [Greenberg et al., 1998; Schoch et al., 2002], was observed in higher concentrations in late post-hypoxic cultures. This might be a reflection of compensatory in vivo processes of revascularisation in our model [Marti et al., 1999; Namiecinska et al., 2005]. VEGF is also known to be involved in neuronal protection through inhibition of Caspase-3 [Gora-Kupilas et al., 2005; Jin et al., 2001]. The time-delayed increased release of VEGF as an effect of hypoxia does not seem to influence the number of apoptotic cells in our cultures. This may be a result of concentrations of VEGF that did not reach efficient thresholds. In this context the measured reduction of caspases on Days 2 and 3 after hypoxia is caused by the already described ongoing energy failure in the post-hypoxic cells. However, the regulation of those apoptotic proteins is probably not related to VEGF mediated-effects.

The therapeutic benefit of cell administration after stroke has been demonstrated in numerous animal studies [Chen et al., 2001; Christensen et al., 2005]. However, important questions in basic cellular and molecular mechanisms of neuronal cell death and its prevention after inadequate oxygen supply still remain unanswered. To understand the mechanisms of cellular neuroprotection after stroke we employed the model of neuronal hypoxia and applied MNC from HUCB.

In vivo MNC are supposed to differentiate into neuronal cells [Chu et al., 2004; Taguchi et al., 2004] to trophically support neuronal tissue through the production of growth factors [Chen et al., 2001], to support the new formation of synapses and migration as well as the differentiation of endogenous neuronal progenitors [Chu et al., 2004; Taguchi et al., 2004]. In our experiments, the administration of MNC from HUCB to post-hypoxic neuronal cultures showed remarkable beneficial effects. Over three days we could show a clear reduction of neuronal apoptosis, even to the level of normoxic control cultures (7% ± 3%). There are two observations that contribute to an explanation of neuroprotective mechanisms. First, MNC were preferentially located close to hypoxically injured neuronal cells. This phenomenon was facilitated through an intensive upregulation of ICAM-1 on neuronal cells which is the specific ligand for leukocyte cell adhesion molecule [LFA-1] expressed by all immune cell subsets [Giblin et al., 2006]. However, the formation of cell chimera was not observed, as there were no CFSE-stained cells with neuronal morphology. In future experiments we will investigate whether blocking of adhesion molecules on the
surface of neuronal cells can inhibit the co-localisation of MNC. Further it will be proved whether this influences the rate of apoptosis. An increase of apoptosis would be an evidence for the significance of direct cell-cell-contacts in context of therapeutic mechanisms of MNC. The second observation would be concomitance of an MNC indicated specific alteration in levels of soluble factors. That change might be held responsible for neuroprotection. Pronounced upregulation of chemokines (CCL5, CCL3, CCL4, CXCL10) might be causal for the enhanced effort of MNC to localise near neuronal structures. High concentrations of VEGF are known to be neuroprotective [Sun et al., 2003; Wang et al., 2006]. In contrast to post-hypoxic mono-cultures, we found no increase of VEGF after administration of MNC. It seems that presence of MNC inhibited the induction of elevated levels of VEGF and compensated its neuroprotective effects by other mechanisms. Cord blood contains distinct cell types capable to differentiate into neuronal cells [Rien-Zakay et al., 2007]. However, the differentiation of stem cells (about 1% within the MNC fraction) was not expected because of the short time of co-cultivation that clearly undershoots time frames responsible for neuronal differentiation.

So far, it is unclear whether neuroprotection resulted from one of the observed effects or from a combination of spatial proximity and specific cytokine patterns. Consequently, further studies in indirect co-cultures will be necessary.
4.6 Conclusions

Pathophysiological models developed from animal studies form the basis of our understanding of the development of stroke. In vivo data display a perfusion-related dependency of neuronal cell damage. Residual energy supply in the penumbra induces apoptosis, the early phases of which are reversible. Consequently, rescue of the penumbra is a major target of experimental stroke therapy. Data obtained from complex animal models of stroke strongly suggest that transplanted cells enhance neuronal survival.

We established a standardized human in vitro model of neuronal apoptotic cell death after hypoxia that can facilitate to address specific pathophysiological processes underlying hypoxic damage and cell-mediated neuroprotection more precisely. Interestingly, transplanted MNC not only strongly decreased the ratio of apoptosis in neuronal cells but also triggered retaining neuronal characteristics such as forming networks. MNC clustered around post-hypoxic neuronal cells and induced an alteration in cytokine and chemokine concentrations. Our data suggest that the neuroprotective effects of MNC might result from direct cell-cell contacts and/or the adjustment of specific soluble mediators.
Based on the observations described in the foregoing paragraph the experiments presented in the following chapter focused on the question that which of the heterogeneous cell fractions composing HUCB has the highest neuroprotective capacity. Therefore, unsorted HUCB-MNC, a stem cell enriched fraction and a stem cell depleted fraction obtained from HUCB-MNC (CD133+ and CD133-, respectively) were either directly or indirectly co-cultivated with post-hypoxic neuronal cells (differentiated SH-SY5Y). Cultures were investigated regarding development of apoptosis and necrosis of neuronal cells, chemotaxis of MNC and production of chemokines and growth factors.

After a co-cultivation time of three days MNC and surprisingly CD133- significantly reduced neuronal apoptosis in both co-cultivation types. In direct co-cultures CD133- produced high concentrations of CCL3 and neuroprotective G-CSF. CD133+ cells effectively reduced neuronal damage in direct co-cultures only.
Chapter 2

6 Neuronal Hypoxia in vitro: Investigation of Therapeutic Principles of HUCB-MNC and CD133+ Stem Cell
6.1 Abstract

Background: The therapeutic capacity of human umbilical cord blood mononuclear cells (HUCB-MNC) and stem cells derived thereof is documented in animal models of focal cerebral ischemia, while mechanisms behind the reduction of lesion size and the observed improvement of behavioral skills still remain poorly understood.

Methods: A human in vitro model of neuronal hypoxia was used to address the impact of total HUCB-MNC (tMNC), a stem cell enriched fraction (CD133\(^+\), 97.38\% CD133-positive cells) and a stem cell depleted fraction (CD133\(^-\), 0.06\% CD133-positive cells) of HUCB-MNC by either direct or indirect co-cultivation with post-hypoxic neuronal cells (differentiated SH-SY5Y). Over three days, development of apoptosis and necrosis of neuronal cells, chemotaxis of MNC and production of chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9) and growth factors (G-CSF, GM-CSF, VEGF, bFGF) were analyzed using fluorescence microscopy, FACS and cytometric bead array.

Results: tMNC, CD133\(^+\) and surprisingly CD133\(^-\) reduced neuronal apoptosis in direct co-cultivations significantly to levels in the range of normoxic controls (7\% \pm 3\%). Untreated post-hypoxic control cultures showed apoptosis rates of 85\% \pm 11\%. tMNC actively migrated towards injured neuronal cells. Both co-cultivation types using tMNC or CD133\(^-\) reduced apoptosis comparably. CD133\(^-\) produced high concentrations of CCL3 and neuroprotective G-CSF within indirect co-cultures. Soluble factors produced by CD133\(^+\) cells were not detectable in direct co-cultures.

Conclusion: Our data show that heterogeneous tMNC and even CD133-depleted fractions have the capability not only to reduce apoptosis in neuronal cells but also to trigger the retaining of neuronal phenotypes.
6.2 Background

Transplantation of adult stem cells has been shown to be an auspicious and effective treatment for degenerative and traumatic neurological diseases [Serakinci et al., 2006]. Among degenerative neurological disorders acute ischemic stroke is the leading cause of disability and death in industrial nations [Egan et al., 2000; Hinkle et al., 2007; Meister et al., 2000].

Acute stroke leads to an increased release of hematopoietic stem and progenitor cells from bone marrow into peripheral blood [Paczkowska et al., 2005]. It is assumed that these cells take part in self-healing processes occurring after neuronal injury. They are supposed to promote the survival of the injured brain tissue by producing neurotrophic factors [Newman et al., 2006], to enhance endogenous angiogenesis [Taguchi et al., 2004] and neurogenesis [Chu et al., 2004] or even to transdifferentiate into neuronal cells [Lu et al., 2002]. However, the stroke induced endogenous release of hematopoietic stem and progenitor cells seems not to be sufficient to compensate massive loss of brain tissue after extended ischemic stroke. Therefore, external application of hematopoietic stem and progenitor cells is expected to complement current treatment of acute stroke based on thrombolytic therapy. An appropriate source of hematopoietic stem cells is the mononuclear cell (MNC) fraction of human umbilical cord blood (HUCB) [Goldstein et al., 2007, Sanchez-Ramos et al., 2006; Weiss et al., 2006]. Transplantation of HUCB-MNC as well as enriched HUCB hematopoietic stem cells into animals which were subjected to focal stroke caused by middle cerebral artery occlusion (MCAO) ameliorated the animals’ functional outcome and reduced the lesion size [Chen et al., 2001]. However, there are still manifold unanswered questions addressing the beneficial influence of such grafts on injured neuronal cells.

It has been documented that there is no neuronal transdifferentiation of hematopoietic stem cells in vitro [Bossolasco et al., 2005; McGuckin et al., 2004; Reali et al., 2006]. Though so far there is no convincing proof that locally administered hematopoietic stem cells transdifferentiate into functionally neuronal cells forming the basis of the animals’ behavioral progression [English et al., 2006]. It has recently been shown that there is no need for MNC to enter the brain for neuroprotection. Soluble factors like GDNF, NGF, BDNF or G-CSF are known to promote neuroprotection over long-distances [Borlongan et al., 2004; Schabitz et al.,]
2003]. This raises many questions about the cellular mechanisms causing the functional improvement after grafting [Arvidsson et al., 2002]. Prevention of neurons from apoptotic cell death [Ferrer et al., 2006] is considered to be supported by the transplantation and could be directly connected to improved tissue conservation, lesion size reduction and superior functional outcome [Romero et al., 2002].

Cell culture models of neuronal hypoxia complement the exploration of particular interactions between grafts and neuronal tissue. Our study is based on a well established post-hypoxic neuronal cell culture model (SH-SY5Y). This model was used to address (i) the neuroprotective potential of stem cell enriched and -depleted HUCB derived cell fractions, (ii) the impact of these cells especially on apoptotic status of oxygen-deprived neurons, and (iii) the mediation of cell-derived survival signals (soluble or cell-attached).
6.3 Methods

6.3.1 Preparation of HUCB samples and isolation of CD133+ cells
HUCB samples of healthy full-term neonates were obtained in accordance with ethical prescripts immediately after delivery. Samples were processed and analyzed as described previously [Hau et al., 2008]. The total MNC (tMNC) fraction gained from Ficoll density gradient (Tab. 1) was stored in the gaseous phase of liquid nitrogen. Cellular sub-fractions of tMNC were characterized using CD3-Phycoerythrin (PE, Immunotech, Hamburg, Germany), CD14-Fluorescin isothiocyanate (FITC), CD16+56-PE (both, Becton-Dickinson, Franklin Lakes, NJ, USA), CD19-Allophycocyanin (APC) and CD45-FITC, (both, Beckman Coulter, Krefeld, Germany). Prior to use tMNC were thawed and stained with carboxy fluoresceindiacetate succinimidyl ester (CFSE, Invitrogen, Karlsruhe, Germany).
CD133 positive cells (CD133+) were isolated from HUCB-MNC using the MACS® immunomagnetic positive selection protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). The flow-through fraction was collected as negative fraction (CD133−) depleted of CD133+. Final populations were analyzed using a FACSCalibur flow cytometer equipped with the CellQuest™ software (both Becton-Dickinson, Franklin Lakes, NJ, USA) and characterized by the use of the following antibodies: CD133/2-PE, Miltenyi Biotech Bergisch Gladbach, Germany), CD45-FITC and CD34-APC (both Beckman Coulter, Krefeld, Germany). Isotype-identical monoclonal antibodies served as controls. CD133+ and CD133− fractions contained 97.38% and 0.06% CD133 positive cells, respectively.
Status of activation of tMNC and CD133− was analyzed via FACS using the antibodies CD25-PE, HLA-DR (Major Histocompatibility Complex, class II, cell surface receptor)-APC (both Becton-Dickinson, Franklin Lakes, NJ, USA), CD38-PE and CD71-FITC (both Beckman Coulter, Krefeld, Germany).
Table 1: Cellular sub fractions of MNC

<table>
<thead>
<tr>
<th>Cellular fractions of MNC</th>
<th>Markers</th>
<th>Content in tMNC (%)</th>
<th>Content in CD133⁺ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I) Myeloid cells</td>
<td>CD14⁺/CD45⁺</td>
<td>14.1 ± 4.4</td>
<td>19.5 ± 2.4</td>
</tr>
<tr>
<td>II) Lymphocytes (including stem cells)</td>
<td></td>
<td>85.9 ± 4.4</td>
<td>80.5 ± 2.4</td>
</tr>
<tr>
<td>B-lymphocytes</td>
<td>CD19⁺/CD45⁺</td>
<td>12.3 ± 5.5</td>
<td>14.6 ± 0.5</td>
</tr>
<tr>
<td>T-lymphocytes</td>
<td>CD3⁺/CD45⁺</td>
<td>73.0 ± 10.2</td>
<td>76.8 ± 1.9</td>
</tr>
<tr>
<td>NK-cells</td>
<td>CD56⁺/CD45⁺</td>
<td>12.7 ± 4.5</td>
<td>8.3 ± 1.4</td>
</tr>
<tr>
<td>Hematopoietic stem/progenitor cells</td>
<td>CD133⁺/CD34⁺</td>
<td>1.2 ± 1.0</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>CD133⁺/CD34⁻</td>
<td>0.4 ± 0.2</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CD133⁻/CD34⁺</td>
<td>0.4 ± 0.2</td>
<td>0.34 ± 0.06</td>
</tr>
</tbody>
</table>

Cellular fractions of HUCB-MNC after thawing of cryopreserved cells. Subpopulations of lymphocytes and stem/progenitor cells are expressed as percent of total lymphocytes.

6.3.2 Differentiation and hypoxic induction of SH-SY5Y cells

For differentiation 0.9 × 10⁴/cm² SH-SY5Y cells were seeded in 16-mm-diameter wells (Greiner Bio-One, Frickenhausen, Germany). After 5 days Dulbecco's Modified Eagle Medium (DMEM) /10 μM all-trans retinoic acid (Sigma-Aldrich, Steinheim, Germany) /15% fetal calf serum (PAN-Biotech, Aidenbach, Germany) media was changed into DMEM-Ham’s F12/10 μM RA/5 ng/ml Brain Derived Neuronal Factor [BDNF, Immunotools Biolance GmbH, Hanover, Germany] /0.1% Human Serum Albumine [HSA, PAN Biotech GmbH, Aidenbach, Germany] for another 11 days. Media were exchanged every third day. Afterwards fully matured neuronal cells were cultured under a hypoxic atmosphere (< 1% O₂) for 48 hours [Hau et al., 2008]. Number of viable cells remained stable between Day 16 and Day 21.

6.3.3 Direct and indirect co-cultivation of post-hypoxic neuronal cells with tMNC, CD133⁺ and CD133⁻ cell fractions

Subsequent to hypoxia, direct and indirect co-cultivation with tMNC, CD133⁺ or CD133⁻ was carried out under normoxic conditions over a period of three days. Added tMNC, CD133⁻ (4.5 × 10⁵ cells, both) and CD133⁺ (4.5 × 10³ cells) were dissolved in 500 μl co-culture medium (DMEM-Ham’s F12, 5 ng/ml BDNF and 0.1% HSA) and added to differentiated post-hypoxic neuronal SH-SY5Y cells cultivated in adequate volume of post-hypoxic medium. Cell ratio of post-hypoxic neuronal cells to
MNC was 1:15 and to CD133$^+$ 1:0.15. For indirect co-cultivation tMNC or CD133$^-$ were added in cell impassable cell culture inserts with a pore size of 0.4 µm (Greiner Bio-One GmbH, Frickenhausen, Germany).

Prior to co-cultivation with post-hypoxic neuronal cells, tMNC as well as CD133$^+$ and CD133$^-$ were labeled with CFSE.

6.3.4 Cell viability assay of neuronal cells
Within direct and indirect co-cultures and control cultures the influence of tMNC, CD133$^+$ and CD133$^-$ on neuronal viability was detected via i) Propidium iodide (PI, Invitrogen, Karlsruhe, Germany) assay for necrosis and late apoptosis and ii) annexin-V assay (Becton-Dickinson, Heidelberg, Germany) for apoptosis. The PI– and annexin-V assays were performed in cell culture plates as described previously [25]. tMNC, CD133$^+$ and CD133$^-$ were distinguished from annexin-V-PE or PI positive neuronal cells by the green CFSE staining.

Cytometric Bead Array for human apoptosis (CBA, Becton Dickinson, Erembodegem, Belgium) was used to quantify the apoptosis specific parameter cleaved Poly-ADP-Ribose-Polymerase I (PARP) in lysates of post-hypoxic neuronal cells after indirect co-cultivation with tMNC and CD133$^-$. For cell lyses adherent neuronal cells were rinsed with PBS and incubated on ice in the supplied buffer for 20 minutes. For analyses pooled samples obtained from three independent experiments were used.

6.3.5 Cytokine profiling
For cytokine profiling supernatants of direct and indirect co-cultures were analyzed on Day 3. Supernatants of tMNC, CD133$^+$ and CD133$^-$ mono-cultures and those of post-hypoxic neuronal cells were also investigated on Day 3. Cytokines were simultaneously measured using CBA for human soluble proteins (Becton Dickinson, Erembodegem, Belgium).

Supernatants were screened for the following cytokines: CCL2, CCL3, CCL5, CXCL8, CXCL9 and for the growth factors basic Fibroblast Growth Factor (bFGF), Granulocyte Colony-Stimulating Factor (G-CSF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and Vascular Endothelial Growth Factor (VEGF). The detection limit was 20 pg/ml, except for CCL2 and VEGF (40 pg/ml).
6.3.6 Statistical analyses of data
Except for apoptosis and necrosis rates all results have been reported as mean ± SD. Statistical differences were analyzed by Student’s t-test or Mann-Whitney rank sum-test. P values of ≤ 0.05 were considered statistically significant (* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001). Apoptosis and necrosis rates were logit-transformed to obtain normally distributed quantities. The effects of time, experimental setting (post-hypoxia), experimental run and the investigated well were determined univariately, and, finally multivariately using a mixed-model approach with time and experimental setting as fixed effects and well and experimental run as random effects.
Cytokine concentrations of indirect and direct co-cultures with tMNC, CD133⁻ and CD133⁺ were compared with the sum of the concentrations obtained after post-hypoxia of neuronal cells and corresponding tMNC, CD133⁺ and CD133⁻ monocultures using a bootstrapping algorithm. Therefore, we added concentrations which were resampled from e.g. the experiment post-hypoxia and the tMNC mono-culture and compared the results with concentrations obtained from e.g. the experiment of indirect co-culture with tMNC. Results were compared with Student’s t-test or Mann-Whitney rank sum test. P-values reported are based on 10,000 bootstrapping simulations. We accounted for multiple comparisons of cytokine concentrations by using Bonferroni’s correction of significance level.
Box plots (if applicable) and univariate analyses were determined using the software package SPSS (SPSS Inc., Chicago IL, USA). Mixed Model analyses were performed using PROC MIXED of the statistical software package SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Bootstrapping analysis was using the statistical software package “R” [Holopainen et al., 2005; Hossman, 2006].
6.4 Results

6.4.1 Direct co-cultivation with each fraction of HUCB-MNC reduced apoptosis in post-hypoxic neuronal cells

Hypoxic cultivation (48 hours) of fully matured neuronal SH-SY5Y cells resulted in an initial rate of apoptosis of 26% ± 13%. Within the following three days rate of apoptosis increased to 85% ± 11%. By contrast, normoxic control cultures showed a stable amount of apoptotic cells (7% ± 3%) over the whole observation time (data not shown). Direct co-cultivation with tMNC and CD133- showed pronounced reduction of neuronal apoptosis. Similar results were obtained after application of CD133+. By application of 4.5 x 10³ CD133+ they were given in equal amounts as they exist in tMNC. Though the whole cell amount of CD133+ was 100 times less then tMNC administered (Fig. 1A).

Levels of necrosis in post-hypoxic control cultures remained nearly stable (approximately 25%) over three days. tMNC and CD133- cell application also induced a significant reduction of necrosis. CD133+ cells did not influence the level of necrosis (Fig. 1B).
Fig. 1: Time course of apoptosis and necrosis of post-hypoxic neuronal cells in direct co-cultures with tMNC, CD133\(^+\) and CD133\(^-\)

Box plots showing time course of apoptosis (A) and necrosis/ late apoptosis (B) in direct co-cultures of post-hypoxic neuronal cells with tMNC, CD133\(^-\) or CD133\(^+\) (colored plots). Grey boxes display the development of cell death in post-hypoxic neuronal cells directly after hypoxic pre-cultivation of SH-SY5Y cells (Day 0) and 1-3 days post-hypoxia. The ordinate represents the percentage of apoptotic and necrotic cells, respectively. Rates of apoptosis and necrosis were calculated in relation to the total number of SH-SY5Y cells.

6.4.2 In indirect co-cultures tMNC and CD133\(^-\) were also sufficient to decrease apoptosis of post-hypoxic neuronal cells

Over the entire observation period, direct as well as indirect co-cultivation with tMNC or CD133\(^-\) exhibited a significant reduction of apoptosis. In all co-culture set-ups percentage of annexin-V positive cells was significantly lower (p ≤ 0.001) than in post-hypoxic control cultures (Fig. 2A, B). Direct and indirect co-cultivation of CD133\(^-\) resulted in similar rates of apoptosis continuously below 5% of annexin-V positive cells (Fig. 2B). However, still generating strong neuroprotective effects, the number of apoptotic neuronal cells in indirect tMNC co-cultures was significantly higher than in direct co-cultures at Day 2 and Day 3 (p ≤ 0.001) as shown in figure 2A. Direct co-cultivation with tMNC resulted in a stable level of 6% ± 1% neuronal apoptosis and was therefore significantly lower than in post-hypoxic control cultures (Day 2: 46% ± 20%; Day 3: 85% ± 11%).

When tMNC were indirectly co-cultured neuroprotection was as pronounced as in direct co-cultures on the first day after hypoxia. Two and three days after application
there was still a significant, but compared to control cultures reduced, protective effect in the indirect co-cultures while protection in direct co-cultures was as distinct as on Day 1 (7% ± 8%).

The comparison of both application types using tMNC and CD133´ showed that soluble factors seem to have strong therapeutic potential (Fig. 2A, B).

The positive influence of indirect co-cultivation on the amount of apoptotic cells, revealed by annexin-V detection, was also confirmed by typical patterns in the cleavage of PARP, a late marker of apoptosis (Fig. 3). In indirect co-cultures with tMNC and CD133´ quantities of cleaved PARP were nearly at the same level on Day 1 post-hypoxia. On Day 2 and Day 3 neuroprotection by CD133´ resulted in concentrations of cleaved PARP ranging in the level of the normoxic control (Fig. 3).

Only direct co-cultures of tMNC and CD133´ displayed improved protection from necrosis/ late apoptosis as revealed by Propidium Iodide labeling of post-hypoxic neuronal cells (Fig. 2C, D). Indirect co-cultivation did not reduce the percentage of necrotic/ late apoptotic neuronal cells.
Fig. 2: Effects of direct and indirect application of tMNC and CD133\textsuperscript{-} on apoptosis and necrosis of post-hypoxic neuronal cells

Box plots represent the percentage of annexin-V positive post-hypoxic neuronal cells (A, B) and of PI positive post-hypoxic neuronal cells (C, D) in direct and indirect co-cultures. Co-cultures were performed with tMNC (A, C) and CD133\textsuperscript{-} (B, D). The extents of apoptosis (annexin-V-binding) and necrosis (PI) were analyzed for three days after hypoxia.
Fig. 3: Direct comparison of effects of tMNC and CD133+ on late apoptosis (PARP cleavage)

Bar chart showing concentrations of cleaved PARP in lysates of post-hypoxic neuronal cells which were indirectly co-cultivated with tMNC (grey shaded bars) or CD133+ (black bars). Controls are post-hypoxic mono-cultures (grey bars) and normoxic cultures (white bars). Each bar visualizes pooled samples obtained from three independent experiments.

6.4.3 tMNC localized in close proximity to post-hypoxic neuronal cells

In direct co-cultures many tMNC were found in close spatial relation with post-hypoxic neuronal cells already at Day 1 (Fig. 4B). This became even more evident at later time points (Fig. 4D, F). At Day 3, the vast majority of tMNC was found adjacent to neuronal somata and processes (Fig. 4F). Interestingly, co-cultivation with tMNC seemed to have strong positive effects on the preservation of typical neuronal cell morphology as the formation of branched processes.

In contrast, in normoxic control cultures tMNC were evenly spread throughout the culture dish (Fig. 4A, C, E).
6.4.4 Cytokine secretion patterns induced by direct co-cultivation with tMNC or CD133^− were similar; cytokines produced by CD133^+ were not detectable

In supernatants of direct co-cultures and corresponding mono-cultures we assessed concentrations of CCL2, CCL5, CXCL8, VEGF and bFGF (Fig. 5).

Post-hypoxic neuronal cells secreted the chemokines CXCL8 and CCL2 and the growth factor VEGF. Low amounts of CCL5 and bFGF were detectable, as well. Mono-cultures of tMNC and CD133^− displayed similar growth factor and chemokine secretion patterns: CXCL8 and CCL2 were produced in considerable amounts while CCL5 and bFGF were secreted at low levels. VEGF was not present. None of the investigated soluble factors was detected in supernatants of purified CD133^+ mono-cultures.

In direct co-cultures with tMNC or CD133^− secretion of cytokines was regulated very similar: reduction of CXCL8 (p ≤ 0.001) and VEGF (p ≤ 0.001) and increase of CCL5 (Fig. 5). CD133^+ co-cultivation only influenced VEGF levels, which decreased significantly (Fig. 5).
Fig. 5: Cytokine profile in direct co-cultures with tMNC, CD133+ and CD133- on Day 3

Control: sum of cytokine concentration from single cultured (grey) post-hypoxic SH-SY5Y cells and HUCB-MNC (light green = tMNC, light red = CD133-, not detectable = CD133+). Co-culture: direct co-culture of post-hypoxic SH-SY5Y cells and HUCB-MNC (dark green = tMNC, dark red = CD133-, dark yellow = CD133+). Data are derived from four independent experiments and are expressed as pg/ml. * significant differences in cytokine concentrations of co-cultures compared to control cultures. Note different axis scaling.
6.4.5 Up-regulation of soluble factors in indirect CD133⁻ co-cultures was associated with long term neuroprotection

In a second set of experiments we investigated different effects of direct and indirect co-culturing on cytokine secretion. Therefore, concentrations of CXCL9, CCL3, VEGF, G-CSF and GM-CSF were measured in normoxic and post-hypoxic monocultures, and co-cultures with tMNC and CD133⁻.

Except VEGF neuronal cells did not secrete detectable amounts of either CXCL9, CCL3, G-CSF nor GM-CSF in monocultures. Both, tMNC and CD133⁻ cells expressed CCL3 and G-CSF but only CD133⁻ produced GM-CSF (Fig. 6).

In co-cultures with tMNC direct co-cultivation generated more pronounced effects than indirect co-cultivation, as seen for the relative up-regulation of CXCL9, GM-CSF (both, \( p \leq 0.05 \)) and CCL3 and the relative down-regulation of VEGF. Only direct CD133⁻ co-cultivation had an impact on regulation of CCL3, VEGF and GM-CSF.

Secretion of all three cytokines was markedly decreased compared to controls whereas indirect co-cultivation with CD133⁻ had no effect. In contrast, the secretion of CXCL9 and G-CSF (\( p \leq 0.01 \)) was markedly induced by indirect co-cultivation with CD133⁻. For these cytokines direct co-cultivation showed no effect (G-CSF) or resulted in a remarkably reduced production (CXCL9) as compared to indirect co-cultivation with CD133⁻ (Fig. 6). The experiment also revealed the influence of different HUCB-MNC-derived cell preparations on the cytokine secretion. Most prominent, direct co-cultivation with tMNC significantly increased expression of CCL3 (\( p \leq 0.05 \)) whereas direct co-cultivation with CD133⁻ resulted in a relative reduction of this chemokine (\( p \leq 0.01 \)).
Fig. 6: Effect of co-cultivation system (direct v. indirect) on cytokine secretion during co-cultivation of post-hypoxic neuronal cells with tMNC and CD133<sup>-</sup>.

**Control:** sum of cytokine concentration from single cultured (grey) post-hypoxic SH-SY5Y cells and HUCB-MNC (light green = tMNC, light red = CD133<sup>-</sup>). **Co-cultures:** co-culture of post-hypoxic SH-SY5Y cells and tMNC (direct: dark green, filled; indirect: dark green, striped), co-culture of post-hypoxic SH-SY5Y cells and CD133<sup>-</sup> (direct: dark red, filled; indirect: dark red, striped). Effects are displayed in percent as relative changes to control.

**Mono-cultures:** absolute cytokine concentrations in supernatants of SH-SY5Y normoxic and post-hypoxic mono-cultures (white or grey bars, respectively) and tMNC (light green bars) or CD133<sup>-</sup> (light red bars).

* significant changes to control.
+ significant differences between co-cultivation systems.
6.5 Discussion

It has been previously shown that HUCB derived MNC as well as nearly pure stem cell populations obtained from MNC are able to improve the clinical outcome of animals after MCAO [Vendrame et al., 2004]. In an \textit{in vitro} model of neuronal hypoxia we discern cell populations within MNC being able to improve neuronal survival and disclose potential neuroprotective mechanisms.

First we studied the effects of tMNC application on the apoptotic status of post-hypoxic neurons. We found that direct application of tMNC results in preservation of neuronal morphology based on a constant protection from apoptosis (Fig. 2A).

For \textit{in vivo} experiments it has been described that none or only a minority of systemically administrated cells were detected in the brain while large quantities were found in the spleen, in the lungs and in the blood of the animals. Nevertheless, some studies showed that cell-treatment improved behavioral deficits [Borlongan et al., 2004; Vendrame et al., 2004; Makinen et al., 2006]. This indicates the importance of soluble factors for neuroprotection. Our data obtained from indirect co-cultures strongly support this hypothesis. tMNC application was highly protective, although the anti-apoptotic effect was slightly weakened after two days (Fig. 2A). Probably, soluble factors act as "first aid" messengers while longer protection seems to demand close proximity between tMNC and neuronal cells. The comparison of direct application of tMNC to normoxic and post-hypoxic neuronal cells revealed that only post-hypoxic neuronal cells attracted tMNC (Fig. 4). The clustering of tMNC around neuronal cells could explain the enhanced protection from apoptosis in direct co-cultures with tMNC at Day 2 and Day 3 (Fig. 4, Fig. 2A). Spatial contiguity is possibly associated with higher concentrations of neuroprotective mediators in the micro-environment of injured neuronal cells. Post-hypoxic neuronal cells were shown to significantly up-regulate the adhesion molecule ICAM-1 following hypoxia [Hau et al., 2008]. Hence, ICAM-1 (CD54) expression could underlay the observed co-localization via binding of LFA-1 (CD11) [Huang et al., 2007]. Besides adhesion molecules, chemotaxis could mediate this cellular co-localization. VEGF, known to exert chemotactic effects on monocytes [Moon et al., 2005], was produced by post-hypoxic SH-SY5Y cells (Fig. 6).

To focus the neuroprotective effects of stem cells we enriched or depleted CD133$^+$ cells from HUCB-MNC. Purities of 97.38% for the stem cell preparation and a
reduction of CD133+ up to 0.06% for the stem cell depleted fraction were achieved. In direct co-cultures CD133+ were applied to neuronal cells in quantities of 4.5 x 10^3 resembling 1% of the applied tMNC, since CD133+ account for about 1% of total cell number in tMNC preparations. Neuroprotective capacity of nearly pure CD133+ cells was comparable to tMNC (Fig. 1A). According to our analyses of cytokine production CD133+ cells did not secrete measureable cytokines neither in mono-cultures nor in co-cultures with injured neuronal cells (Fig. 5). Additional experiments using even tenfold elevated numbers of stem cells (4.5 x 10^4) also revealed no detectable cytokine concentrations and did not result in an increased neuroprotective capacity (data not shown). Neuroprotection by the absence of measurable soluble mediators argues for a stem cell specific therapeutic mechanism through contiguity. Due to this assumption we did not include CD133+ in the investigation of indirect co-cultures in this study.

Surprisingly, CD133- cell fractions were also highly sufficient in protection from apoptosis. Therefore, the observed anti-apoptotic neuroprotective effects of tMNC in our experiments do not only relay on hematopoietic stem cell-specific mechanisms. This assumption is supported by the missing significance in the effect of CD133+ on necrotic/late apoptotic loss of post-hypoxic neuronal cells (Fig. 1B). tMNC and CD133- significantly reduced the percentage of PI-positive neuronal cells, whereas CD133+ did not. Since CD133- in indirect co-cultures were superior to tMNC in protection from apoptosis and because of very low amounts of CD133- in this preparation, CD133- seem to mediate additional neuroprotective effects. Possibly, the separation process influenced the functionality of CD133- cells. FACS analyses of the activation markers CD25, CD38, CD71 and HLA-DR on tMNC and on the separated CD133- fraction did not reveal an increased population of activated cells in our preparations (data not shown). However, the expression of other activation molecules cannot be excluded, since this cell population displayed an enhanced secretion of cytokines like G-CSF, GM-CSF and CCL-3 (Fig. 6). We also cannot rule out that remaining CD34+ cells could account for the neuroprotective activity of the CD133- fraction, since depletion did only reduce the number of CD34+ cells to 77% (Tab. 1). The investigation of soluble mediators exhibited that hypoxia induced a significant increase of VEGF in neuronal cells (Fig. 6, [Bergeron et al., 2000]). VEGF is documented to inhibit pro-apoptotic signaling by Bad (BCL2 antagonist of cell death), and cleavage of caspase-3, and caspase-9 [Gora-Kupilas et al., 2005] and therefore
can be claimed as an autocrine self-protection mechanism of damaged neuronal cells. The neuroprotective impact of tMNC and CD133- cells in direct applications was accompanied by prevention of VEGF production which is typically induced in post-hypoxic neuronal cells (Fig. 5).

Neuroprotective effects of cell application could be mediated by G-CSF that was found only in mono-cultures of CD133- but not in mono-cultures of post-hypoxic SH-SY5Y cells. Schneider et al., 2005 [Schneider et al., 2005] pointed out that human SH-SY5Y neuroblastoma cells express the G-CSF receptor and that activation by the neurotrophic G-CSF reduced NO-induced poly-ADP ribose polymerase (PARP) and caspase-3 cleavage. Our data support these observations. At Day 3 in indirect co-cultures supply of G-CSF by CD133- was associated with cleaved PARP levels in the range of normoxic cultures (Fig. 3, Fig. 6). G-CSF levels in mono-cultures of tMNC were only slightly above detection limit and could not provide this anti-apoptotic effect (Fig. 6). The decrease of cleaved PARP observed in post-hypoxic neuronal mono-cultures at Day 3 (Fig. 3) was probably induced by rising lack of energy due to increased rate of apoptosis (Fig. 1). Noticeably, there are application-specific differences in the regulation of cytokine secretion in co-cultures with CD133- fractions. Concentrations of CCL3 and G-CSF were significantly higher in indirect co-cultures than in direct co-cultures. Possibly these enhanced concentrations are responsible for a protection from apoptosis in indirect co-cultures similar to that in direct co-cultures at Day 3. Indirect co-cultivation with tMNC did not induce enhanced cytokine levels (Fig. 6) and at the same time did not exert the same neuroprotective effect on post-hypoxic SH-SY5Y neurons. This could be explained by spatial effects: paracrine released cytokines could be more effective than action of cytokines over a longer distance.


6.6 Conclusions

In this study we investigated human umbilical cord blood derived cell populations (tMNC, CD133+, and CD133-) according to their ability to protect post-hypoxic neuronal cells.

For different reasons, as the missing systemic effects and the disregard of brain cell interactions this in vitro system does only simplified reflect the action of MNC after hypoxic brain lesions in vivo. But taken this into account, our study delivers useful indications for the in vivo application of such cells:

So, since purified CD133+ fractions are not superior to total HUCB-MNC in mediating neuroprotective anti-apoptotic effects, expensive and time consuming stem cell separations are not necessarily needed to yield neuroprotective cell populations. Furthermore, our study underlines the importance of MNC derived soluble factors for the mediation of neuroprotective effects visible as prevention of neuronal cells from apoptosis. Therefore, future therapeutic approaches should focus on the sufficient supply of soluble anti-apoptotic mediators, to reduce post-hypoxic brain damage.
7 Preface to Chapter 3

The not yet published material in the following chapter takes the question on neuroprotective mechanisms of HUCB-MNC to a next level by administrating MNC, CD34- and CD34+ to ex vivo organotypic slice cultures subjected to oxygen and glucose deprivation. Compared to cultures of neuronal cells the utilization of organotypic hippocampal slice cultures allows investigation of MNC mediated effects in a multi cell type tree dimensional network of functional neuronal tissue forming a set up that is sufficiently complex and simple at the same time.

Experiments on efficient cell doses revealed a direct relation in number of deployed MNC and reduction in neuronal damage. Migration assays showed that directly administered MNC actively migrate towards OGD damaged hippocampal slices whereas CD34+ cells showed highest homing activity. One aspect for the observed MNC related reduction in neuronal damage might be a reduced secretion of nerve growth factor in the hippocampal tissue.
8 Human Umbilical Cord Blood Cells Reduce Neural Damage in Oxygen and Glucose Deprived Rat Hippocampal Slices
8.1 Abstract

Transplantation of human umbilical cord blood mononuclear cells (HUCB-MNC) in animal models of stroke elucidated the therapeutic potential of these cells. However, effective cell populations within HUCB-MNC and their modes of action are not yet indentified.

To assess the neuroprotective properties of different HUCB-MNC fractions rat organotypic hippocampal slice cultures were subjected to oxygen and glucose deprivation. HUCB-MNC or fractions thereof enriched (CD45+/CD34+) or depleted (CD45+/CD34−) for CD34-expressing stem and progenitor cells were (i) directly added onto hippocampal slices or (ii) co-cultivated indirectly.

After direct application, HUCB-MNC were able to reduce neural damage in hippocampal slices throughout the full observation period (three days). CD34+ cells provided transient protection for two days, whereas the CD34− fraction failed to reduce neural degeneration. Direct addition of HUCB-MNC was superior to indirect co-cultivation of equal cell numbers (2.5x10^4 cells per slice). Rising concentrations of HUCB-MNC (up to 25x10^4) stepwise enhanced the protective effect to levels comparable to direct application. After direct application HUCB-MNC actively migrated into the slices. Flow cytometric analysis of migrated cells revealed that the CD34+ cells within HUCB-MNC were particularly attracted to supernatants of damaged hippocampal tissue. Analysis of trophic factor secretion indicates that HUCB-MNC-provided reduction of damage is accompanied by reduced nerve growth factor secretion from hippocampal tissue.

The results of this study suggest that CD34+ cells are particularly involved in the protective action of HUCB-MNC after oxygen and glucose deprivation, at least partly by mediation of soluble factors derived by HUCB-MNC. Furthermore, CD34+ cells preferentially home to ischemic neuronal tissue.
8.2 Introduction

Transplantation of human umbilical cord blood (HUCB) mononuclear cells (MNC) for experimental therapy of stroke has been thoroughly investigated since the first description of a therapeutic effect of these cells (Chen et al., 2001). Intravenous administration of HUCB-MNC reduced infarct volume and ameliorated behavioural deficits following middle cerebral artery occlusion in rodents (Vendrame et al., 2004). HUCB populations are proven not to induce severe graft versus host disease in allogenic use. These cells are also among the few stem cell containing populations allowing autologous application and might therefore become of clinical relevance (Brown et al., 2008; Yu et al., 2009). However, therapeutic mechanisms of HUCB transplanted after cerebral ischemia still remain speculative. Systemically delivered HUCB-MNC were identified near the lesion by immunohistochemistry and polymerase chain reaction (Vendrame et al., 2004). Whereas, Chen et al. (2001) only localized 0.3% of administered cells near the ischemic lesion and proof for differentiation into functional neurons e.g. by using electrophysiological methods is still lacking. HUCB-MNC more likely influence the imbalanced neuronal microenvironment following stroke rather than to differentiate into functional brain tissue.

There is growing evidence that HUCB therapy may exert neuroprotection by delivery of anti-inflammatory and immunomodulating molecules. HUCB contains a wide range of cell types that could mediate such effects. Beside potential therapeutic candidates like endothelial progenitors (Hildbrand et al., 2004) and mesenchymal stem cells (Tondreau et al., 2005), HUCB-MNC contain about 1% CD34+/CD45+ hematopoietic stem/progenitor cells as well as lymphoid and monocytic populations (Reich et al., 2008). HUCB-MNC are known to secrete numerous soluble factors as interleukin (IL)-8, macrophage chemoattractive protein-1 (Newman et al., 2006), IL-10 (Rainsford et al., 2002) and nerve growth factor (NGF, Bracci-Laudiero et al., 2003). By providing these factors, therapeutic cells may protect post stroke tissue by limitation of free radicals, inflammatory cytokines, lipases, and peroxidases and by neurotrophic signaling (Sanberg et al., 2005; Newman et al., 2006).

Since the complexity of in vivo models for cell transplantation complicates focussed investigation of cellular interactions we used rat hippocampal slice cultures to determine effects of HUCB-MNC on survival of neurons after oxygen and glucose
deprivation (OGD). OGD of organotypic cultures has been widely proven to mimic many aspects of stroke and therefore is a well established tool to test potential neuroprotective therapies (Sarnowska et al., 2009). Comprising all types of neural cells in a three-dimensional network, hippocampal slice cultures offer the possibility to discriminate certain cellular effects in a sufficiently complex, but at the same time well defined experimental setup. Moreover, host immunologic responses after peripheral allogenic application possibly influencing stroke outcome are also excluded by using organotypic slices.

Here, we investigated HUCB-MNC, CD34+, CD34- fractions, and Jurkat T cells, regarding to their ability to reduce neural cell damage in hippocampal slice cultures after OGD. In this context we addressed the protective potential in relation to application manner, cell number and secretion of soluble factors. We furthermore determined cell-type directed chemoattractive characteristics of damaged hippocampal tissue.
8.3 Materials and methods

8.3.1 Preparation of umbilical cord blood samples
Cord blood samples of healthy full-term neonates were taken in accordance to ethical regulations immediately after delivery. Samples were processed by density gradient separation using Lymphocyte Separation Medium (PAA Laboratories, Cölbe, Germany). The HUCB-MNC fraction suspended in fetal calf serum containing 8% dimethyl sulfoxide (Serumwerke Bernburg Inc., Bernburg, Germany) was cryopreserved in the gaseous phase of liquid nitrogen (Air Liquide GmbH, Düsseldorf, Germany). Prior to use, HUCB-MNC were rapidly thawed in phosphate buffered saline (PBS) supplemented with 75 U/ml DNase I (Roche Diagnostics, Mannheim, Germany; Sigma, Germany) and rinsed in RPMI 1640 (PAA).

CD34\(^+\) cells were isolated from fraction by using the MACS\(^\circledast\) immunomagnetic positive selection protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). Obtained cells were analyzed using a FACSCalibur flow cytometer equipped with the CellQuest\(\text{TM}\) software (both Becton-Dickinson, Franklin Lakes, USA) and characterized by the use of the following antibodies: CD45-fluorescein isothiocyanate (FITC) and CD34-allophycocyanin (APC, both Beckman Coulter, Krefeld, Germany). Isotype-identical monoclonal antibodies served as controls. CD34 enriched (CD34\(^+\)) and CD34 depleted (CD34\(^-\)) fractions contained 94.11\% ± 3.42\% and 0.06\% ± 0.03\% CD34 expressing cells, respectively.

MNC, CD34+ and CD34- cell suspensions were stained (10 minutes at 37 °C) with carboxy fluoresceindiacetate succinimidyl ester (CFSE 5 µM; Molecular Probes, Leiden, The Netherlands) to visually control successful application of cells onto the slices. For migration studies HUCB-MNC were stained with CellTracker Orange (5- and-6- 4-chloromethyl benzoyl amino tetramethylrhodamine 5-chloromethylfluorescein diacetate, CTO, 5 µM; Molecular Probes, Leiden, The Netherlands) according to manufacturer’s protocol.

8.3.2 Experimental animals
All experiments involving experimental animals were conducted according to the Magdeburg Regional Board, governmental commission for animal welfare and in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). Organotypic hippocampal slice cultures were prepared from 60
postnatal Wister rat pups (P7-9, Harlan Winkelmann, Borchen, Germany) as described previously (Stoppini et al., 1991).

For migration experiments including confocal analysis, hippocampal slice cultures were prepared from 15 neonatal transgenic B6.Cg-TgN (Thy1-YFP) 16 Jrs mice (P5-9, Jackson Laboratories, distributed by Charles River, Wilmington, USA). These animals express enhanced Yellow Fluorescent Protein (eYFP) at high levels in subsets of central neurons, including pyramidal cells of the hippocampus (Feng et al., 2000). Briefly, rat and mouse pups were sacrificed by rapid decapitation, a method recommended by the Magdeburg Regional Board. Afterwards, the brain was removed from the skull for ex vivo hippocampus preparation.

8.3.3 Organotypic hippocampal slice cultures
Hippocampi were dissected and transversely sliced (350 µm) on a McIlwain tissue chopper (The Mickle Laboratory Engineering, Guildford, UK). Slices were then carefully transferred onto humidified 0.4 µm porous Millicell membrane inserts (Millipore, Mosheim, France). Cultures were maintained in 1 mL of serum-based medium (50% MEM-Hanks, 25% HBSS, 17 mM HEPES, 5 mM glucose, 1 mM L-glutamine, 25% horse serum and 0.5% gentamycin) at 37°C for three days. Thereafter, cultures were transferred to serum-free medium (50% MEM-Hanks, 25% HBSS, 17 mM HEPES, 5 mM glucose, 1 mM L-glutamine, 25% Neurobasal-A, 0.5% B27 and 0.5% gentamycin; referred as standard condition) and kept for 7 days at 37°C in 5% CO₂ with medium change twice a week. Twenty four hours before experimental use, 2 µL propidium iodide (PI; 1 mg/mL, Sigma, Deissenhofen, Germany) was added to the culture medium. Organotypic hippocampal slices were then screened by fluorescence microscopy to exclude cultures that were pre-damaged during the preparation process.

8.3.4 Oxygen and glucose deprivation injury
For OGD, hippocampal slices were transferred to six-well-plates containing 1 mL of 10 mM mannitol-containing glucose-free Ringer solution. Cultures were placed in a gas-tight chamber at 37°C. Oxygen was replaced by 95% N₂ and 5% CO₂ within 10 minutes and slices were incubated for another 40 minutes. Untreated control cultures were maintained for the same time under a normoxic atmosphere in glucose-containing Ringer solution.
8.3.5 Co-culturing of hippocampal slices and HUCB cell fractions
After OGD slices were returned to normoxic standard culture conditions. Afterwards, 1 µL Neurobasal A medium containing 2.5x10^4 HUCB-MNC (CTO-labelled for migration studies), CD34^+, CD34^- cells or Jurkat T cells was administered directly onto the slices. For indirect co-culturing, 2.5x10^4, 12.5x10^4 or 25x10^4 HUCB-MNC per slice were seeded under the interface cultures. Direct and indirect co-cultures were analyzed on three consecutive days. The experimental set ups for OGD and co-culturing are shown in supplementary figures 1 and 2, respectively.

8.3.6 Analysis of cellular damage
After one, two or three days slices were incubated with 10 µl PI (1 mg/mL) for two hours. Damage was analyzed in the cornu ammonis (CA1-CA2-CA3). Propidium iodide fluorescence was elicited at 546 nm and recorded at >610 nm on an inverted Nikon Eclipse TE-2000 fluorescence microscope (4-fold magnification; Nikon, Duesseldorf, Germany) and subsequently analyzed using an image analysis software (LUCIA M, Nikon). To ensure comparability of data, in each individual experiment fluorimetric mean values obtained at Day 1 from slices that underwent OGD were defined as 100%. All other data obtained are given as relative values.

8.3.7 Descriptive migration assay
For descriptive migration assay, HUCB-MNC were stained with CTO as stated above. Migration studies were performed using OGD slices and normoxic control slices after 3 days of maintenance using a Zeiss LSM Pascal 5, Laserlinie 488 confocal laser scanning microscope (Zeiss Plan Neofluar 20x or 40x, Jena, Germany).
8.3.8 Quantitative flow cytometric analysis of migrating HUCB-MNC

For quantitative migration assessment, slices were collected and stored at -80°C until further use. After thawing, slices were homogenized using 70 µm cell strainers (BD Falcon, Franklin Lakes, USA). Extracts were dissolved in serum-free medium and protein concentration was adjusted to 1 µg/ml. 500 µl homogenate solution were filled in the lower compartment of the migration chambers. Equal volumes of medium supplemented with 200 ng/ml recombinant Stromal Cell-Derived Factor (SDF; Immunotools, Friesoythe, Germany) served as controls. 1x10⁶ HUCB-MNC were suspended in 100 µl medium and administrated into the upper part of the migration chamber build by 8 µm pore size cell culture inserts (Greiner, Frickenhausen, Germany). Cells were allowed to migrate for four hours in a humidified incubator at 37°C and 5% CO₂. Afterwards, inserts were removed and migrated HUCB-MNC were collected. Viability and numbers of migrated cells were determined using the Trypan Blue exclusion method and BD Truecount Tubes (BD Biosciences, San Jose, CA, USA), respectively. HUCB-MNC were characterized according to cellular subtypes by use of a FACSCalibur flow cytometer (Becton Dickinson) using the following sets of antibodies: (i) CD45-APC, CD19-phycoerythrin-cyanin 7 (PE-Cy7, both Beckman Coulter), CD14-FITC (Becton Dickinson), CD3-PE (Immunotech, Hohenlockstedt, Germany), (ii) CD45-PE-Cy7, CD133/2-PE, CD34-APC (Beckman Coulter), (iii) CD3-FITC, CD56-PE (Becton Dickinson). Isotype-identical monoclonal antibodies served as controls.

8.3.9 Analysis of human and rat soluble mediators

To profile human and rat trophic factors supernatants of co-cultures were investigated using antibody array kits (RayBiotech, Norcross, USA). Membranes for analysis of soluble factors were plotted with antibodies against human brain derived neurotrophic factor (BDNF), NGF, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1); or against rat ciliary neurotrophic factor (CTNF), NGF and VEGF. Supernatants were pooled from six wells, each containing three slices. Samples were centrifuged (400 x g, 10 minutes) and stored at -80°C. Double concentrated (3400 x g, 20 minutes, Amicon Ultra-4, Millipore, Eschborn, Germany) supernatants from OGD cultures as well as supernatants after direct (2.5x10⁴) and indirect (2.5x10⁴, 12.5x10⁴, 25x10⁴)
application of HUCB-MNC were investigated. Array was performed according to manufacturer's instructions. Chemiluminescence of membranes was visualized in the Intelligent Dark Box II (Fuji Photo Film, Düsseldorf, Germany) for 40 seconds.

8.3.10 Statistical analysis of data
All results are reported as mean ± standard deviation except for data on HUCB-MNC migration and rat soluble factors which, for methodological reasons, represent pooled samples. Statistical differences were analyzed using Mann-Whitney rank sum-test expect experiments on migration where Student’s t-test was used. P values of <0.05 were considered statistically significant and are indicated by single asterisks or alternative symbols (*, or # and §).
8.4 Results

8.4.1 Reduction of OGD induced damage in hippocampal slices after HUCB-MNC application

In a first set of experiments we evaluated the protective properties of different cell preparations derived from human cord blood (HUCB-MNC, CD34⁺, CD34⁻) and of a T cell line by semiquantitative analysis of PI staining after OGD. OGD procedure induced broad cellular damage, predominantly in the pyramidal layer of hippocampal slices, which was indicated by a strong increase in PI staining. The degree of damage remained stable throughout the whole exploration period (Fig. 1A and Fig. 1B upper vs. middle row). Direct application of HUCB-MNC had a significant protective impact (p<0.05) and reduced cellular degeneration by above 50% at every investigated time point (Fig. 1A, B lower row).

Comparable to HUCB-MNC, the CD34⁺ fraction reduced cell degeneration at Day 1 and Day 2 but failed to provide protection effect on Day 3. On the other hand, depletion of CD34 expressing cells (CD34⁻) from HUCB-MNC abolished reduction of neural damage, as indicated by higher PI staining. When applied on damaged hippocampal slices Jurkat T cells also did not reduce neural degeneration. Application of those cells was even associated with exacerbation of OGD induced cellular on Day 3 (p<0.05; Fig. 1A).
Figure 1. Evaluation of neuroprotective properties of HUCB-MNC, CD34⁺, CD34⁻ and Jurkat T cells following OGD on rat hippocampal slices

After OGD, 2.5x10⁴ cells of each fraction were directly applied onto rat hippocampal slices. Cellular degeneration in the cornu ammonis was assessed by PI staining for three subsequent days. Application of HUCB-MNC and CD34⁺ cells diminished damage at Days 1 and 2 (p<0.05, Mann-Whitney-Test) as indicated by reduced PI fluorescence intensity, but only HUCB-MNC were able to provide protection until the end of the observation period (A). CD34⁻ cells failed to prevent cell damage in the cornu ammonis while the application of T cells even raised the amount of cellular damage at Day 3 (p<0.05, Mann-Whitney-Test). White bars represent PI fluorescence in normoxic control hippocampal slices. All bars represent relative mean values ± SD of PI fluorescence assessed in three separate experiments comprising six slices each (n=18). Fluorimetry values obtained from OGD slices at Day 1 were set 100%.

Representative fluorescence micrographs of rat hippocampi cultivated under normoxic conditions, after OGD, and after OGD followed by HUCB-MNC application are given in (B).
8.4.2 Influence of OGD damaged hippocampal tissue on migration of HUCB-MNC fractions

MNC were labelled with CTO and applied onto mouse slices with transgenic neuronal expression of eYFP (Fig. 2). Fluorescence microscopic evaluation revealed that after application of 2.5x10^4 HUCB-MNC onto damaged tissue more cells adhered to the slice as compared to application of the same HUCB-MNC number on normoxic hippocampal tissue (Fig. 2A vs. 2B). In addition, three-dimensional confocal analyses showed that HUCB-MNC preferentially invaded into the damaged nervous tissue (Fig. 2D and 2E). In contrast, HUCB-MNC predominantly remained at the slice surface when applied onto normoxic control slices (2E vs. 2F). Cocultivated with normoxic control slices slices, HUCB-MNC remained morphologically unchanged, whereas applied onto OGD slices, HUCB-MNC often converted from round to enlongated phenotype (Fig. 2C).

A migration assay using supernatants of homogenized organotypic cultures (Newman et al., 2005) was used to obtain information about the cell population migrating into lesioned hippocampal tissue. In this assay undamaged normoxic control tissue and tissue that underwent OGD prior to homogenization induced migration of comparable cell numbers cells (Fig. 2G), each of them significantly differing from medium control (p<0.05). Addition of SDF-1 (CXCL-12), a potent chemoattractant for hematopoetic stem and progenitor cells (Aiuti et al., 1997), monocytes and lymphocytes (Bleul et al., 1996), markedly induced migration of HUCB-MNC towards the lower chamber of the culture system (p<0,01; Fig. 2G), thus serving as positive control in this assay.

Migrated cells where analyzed by flow cytometry to investigate the chemotactic influence of damaged nervous tissue on different HUCB-MNC fractions (T cells, B cells, NK cells, CD34^+, and CD133^+ cells). Comparison of the total numbers of different migrated cells revealed no obvious differences between OGD slices and normoxic control tissue. In all setups, T cells were the most prominent fraction within migrated HUCB-MNC (Fig. 2H, note logarithmic scale). Relative comparison between originally applied cell numbers and migrated cells for every of the investigated cell population (see above) revealed that, in difference to normoxic tissue (control), OGD damaged hippocampal tissue preferentially attracted CD34^+ stem/progenitor cells (Fig. 2I). Attraction of other cell fractions did not markedly differ between control and damaged hippocampal tissue extracts.
Figure 2. Induction of active migration of CD34⁺ cord blood cells by post ischemic hippocampal tissue

A-F (results obtained from transgenic mice): When 2.5x10⁴ CTO-labelled, HUCB-MNC (red) were added directly onto hippocampal slices of B6.Cg-TgN, Thy1-eYFP 16 Jrs transgenic mice (green fluorescence in pyramidal neurons) after three days of cultivation, only scattered, round HUCB-MNC were seen on control slices (A). In contrast, numerous HUCB-MNC were observed on OGD slices (B). These cells preferentially developed an elongated soma (C, insert in B). More cells migrated into the tissue (D, E) as compared to normoxic control slices (F). CTO signals from HUCB-MNC in the slices are indicated by white arrow heads. Scale bars represent 100 µm in (A) and (B) and 25 µm in (C) and (D). D shows total z view of an OGD slice. Inserts in E and F represent vertical sections at positions indicated by green or red lines.

G-I (results obtained from rat hippocampal slices): Cell free supernatants were obtained from homogenates of 23 rat hippocampal slices after three days of cultivation and added to the lower compartment of a migration chamber. Migration of HUCB-MNC towards supernatants was investigated and compared to medium and medium supplemented with SDF-1 as negative and positive controls, respectively (n=6) (G). Hippocampal slices with and without previous OGD induced migration of similar numbers of HUCB-MNC (G; p<0.05 as compared to medium control, Student’s t-test), while strongest migration was observed towards SDF-supplemented medium (G, p<0.001, triple asterisks, Student’s t-test). However, absolute and relative number of migrated CD34⁺ cells was higher after OGD (H and I) as compared to slices maintained under normoxic conditions. Note logarithmic scaling of ordinate in H.

8.4.3 Effects of application mode and cell dose on neurodegeneration in hippocampal slice cultures

After HUCB-MNC application appeared to be highly effective we aimed to evaluate the role of direct cell contacts for mediation of the protective potential after OGD. For that reason effectiveness of directly applied HUCB-MNC was compared with indirect
application, the latter only allowing transposition of soluble factors, but not direct cell-cell contact between damaged hippocampal tissue and HUCB-MNC.

Indirect cocultivation of the same number of HUCB-MNC (2.5x10^4) had a markedly lower protective effect as revealed by PI-staining. In contrast to direct application, this effect could only be discriminated from spontaneous neurodegeneration in untreated OGD slices at Day 2 (p<0.05; Fig. 3A). To test whether this less pronounced effect could potentially be caused by lowered concentrations of soluble neuroprotective factors after indirect HUCB-MNC application, rising numbers of cell were applied to OGD damaged slices (Fig. 3B). Five-fold elevation of HUCB-MNC number (12.5x10^4) diminished degeneration at all time points investigated following OGD (p<0.05; Fig. 3B). At Day 2 after OGD, a further increase of applied HUCB-MNC number (25x10^4) caused an additional significant reduction of PI-staining (Fig. 3B) indicating a dose-depending protection by indirectly applied HUCB-MNC.
Figure 3. Dose-dependent reduction of cellular damage after indirect application of HUCB-MNC onto rat hippocampal slices

For indirect application, HUCB-MNC were placed under the slice culture inserts. Slices were observed for three days. In contrast to direct application, 2.5x10^4 HUCB-MNC were only transiently able to diminish cellular damage in hippocampal slices following OGD (A, p<0.05, Mann-Whitney-Test) and failed to provide cellular protection at Days 1 and 3 (A, p=0.189, Mann-Whitney-Test). Indirect co-cultivation using 5- and 10-fold increased cell numbers was able to reduce OGD induced neural degeneration on all days (p<0.05, Mann-Whitney-Test). The protective effect of indirect HUCB-MNC application gradually increased with rising cell concentrations, in particular at Days 1 and 2 (B). Bars show the mean ± SD of three independent experiments each comprising six slices (n=18). Values were set in relation to OGD at Day 1 which was set 100%. (*) Significantly different from OGD; (#) from 2.5x10^4 HUCB-MNC indirect, and ($) from 12.5x10^4 HUCB-MNC indirect.

8.4.4 Expression of neurotrophic factors

Since evaluation of indirect application of HUCB-MNC indicated a potential role for soluble protection providing agents, HUCB-MNC induced changes in the expression of human and rat derived trophic factors were analyzed. Changes in the expression of human trophic factors secreted by applied HUCB-MNC were not detectable because concentrations of measured human factors (NGF, EGF, IGF-1, BDNF) were below the detection limit of the assay (40 pg/ml). VEGF secretion from rat hippocampal tissue did not change after direct or indirect application of human HUCB-MNC (Fig. 4, left). Evaluation of rat NGF levels revealed a reduction of NGF secretion from OGD slices after application of HUCB-MNC in all co-cultivation
paradigms (Fig. 4, right). Rat CNTF was not secreted in measurable amounts. Since analysis had to be carried out with pooled samples to reach detection limits, descriptive statistics were not applicable.

**Figure 4. Expression of neurotrophic factors**

Rat neurotrophic factors were measured in supernatants obtained from six pooled rat hippocampal slices that were co-cultivated with human HUCB-MNC. Bars illustrate relative intensity units. Evaluation of VEGF secretion from rat hippocampal tissue did not reveal changes, but NGF secretion was reduced in all performed co-cultivation setups. Values obtained from normoxic control slices without HUCB-MNC were set 100% and are indicated as dashed line.
8.5 Discussion

MNC preparations from HUCB have been emerged as a highly promising tool for protection of neural tissue and for enhancement of regeneration following stroke. However, the mode of action underlying the therapeutic potential of HUCB-MNC is still a matter of debate. Some investigators found evidence for limited replacement of lost neuronal cells after ischemia (Chen et al., 2001). Stem cells contained in HUCB-MNC are in principle able to transdifferentiate into neural cells (Greschat et al., 2008) but their functionality *in vivo* still remains unproven. Other results indicate that HUCB-MNC could limit the progress of neuronal degeneration by delivering trophic factors (Arien-Zakay et al., 2009), by modulation of the neuroinflammatory response (Vendrame et al., 2005) and by activation of anti-apoptotic pathways (Dasari et al., 2008).

8.5.1 Ischemic damage reduction by HUCB cell populations

To address the question how HUCB-MNC may diminish ischemic damage of nervous tissue we first analyzed the neuroprotective properties of different cell types within HUCB-MNC preparations. Neuronal loss in OGD damaged hippocampal tissue indicated by PI staining was reduced over 3 days only by the whole HUCB-MNC fraction. Purified CD34+ stem and progenitor cells provided protection on the Day 1 and 2, but not thereafter. CD34 depleted HUCB-MNC (CD34-) abolished the observed reduction of PI-staining (Fig. 1).

These results indicate that CD34+ cells might preferentially trigger the retaining of OGD damaged neuronal tissue. Comparison of the different results obtained with HUCB-MNC and CD34+ on Day 3 suggests that another cell type within both cell preparations could protective the effect maintenance (Fig. 1). Therefore, the main fraction within HUCB-MNC, CD4+ T cells, was taken into account (Brown et al., 2008) by using the Jurkat T cell line that is characterized by expression of CD4 and secretion of interferon gamma (Hayashi et al., 2008) as a surrogate.

Though, the T cell line was not able to provide neuroprotection and even exacerbated tissue damage (Day 3). This argues against activated Th1 biased CD4 T cells as mediators of tissue protection and is in accordance with animal models of focal stroke. In those proinflammatory activated T cells exacerbated tissue damage
that probably lead to proinflammatory activation of microglial cells (Chabot et al., 2001).

The results obtained in the present study differ from data that we previously gained, using a post hypoxic neuronal cell culture model based on a fully differentiated a neuroblastoma cell line. In that experimental setup, both, stem cell enriched and stem cell depleted HUCB-MNC had strong protective capacity (Reich et al., 2008). Such divergent results obtained in two neuronal cell culture models indicate a strong system dependency, which always should be taken into consideration, while interpreting *in vitro* results. A major difference that could explain these variations in results is the preserved tissue architecture in organotypic hippocampal slices including functional neuronal contacts and the presence of glial cells. Results obtained by our group with the same organotypic system demonstrated that for instance microglia can enhance neuronal survival when applied without previous proinflammatory commitment by LPS (Neumann et al., 2006).

### 8.5.2 Protective effects of HUCB-MNC after direct and indirect application to OGD OHC

Two different experimental paradigms were used in this study: (i) direct application of HUCB-MNC and (ii) co-cultivation of hippocampal slices and HUCB-MNC separated by a porous but cell dense membrane that allows detection neurotrophic effects provided by soluble HUCB-MNC derived factors. HUCB-MNC co-cultivated separately from neuronal tissue provided diminished protection of neural tissue compared to direct application of equal cell numbers. This observation could be explained by concomitant action of soluble factors and cell contact effects. Possibly, the diminished effects were caused by reduced concentrations of soluble mediators when cells where placed remote from post ischemic tissue. To test the latter we used rising numbers of HUCB-MNC in the indirect co-cultivation setup. With this approach we could reach protective effects comparable to direct application, only occurred by using 5- and 10-fold increased cell numbers (Fig. 3B). This dose-dependency further points to of soluble factors as main mediators of the reduced neural damage after OGD.

This observation bears two practical consequences. First, the data imply peripheral intravenous application of cells as potential therapeutic strategy after stroke. Second, therapeutic effects would depend on sufficient concentrations of protection providing
substances at the site of action. Therefore, directed accumulation of systemically
applied cells should enhance its therapeutic effect.

8.5.3 Migration of HUCB-MNC towards OGD damaged OHC

Migration studies showed that HUCB-MNC invaded the hippocampal tissue primarily
after OGD (Fig. 2). The elongated morphology of invaded cells implies an active
migration process (Ridley et al., 2003). To dissect subtypes of HUCB-MNC
preferentially migrating towards the OGD damaged hippocampal tissue solution we
employed a migration chamber and characterized migrated cells according to surface
markers for T cells, B cells, NK cells, CD45/CD34+, and CD45/CD133- cells. We
used SDF-1 (CXCL12) known to be strong regulator of cell recruitment as a positive
control to demonstrate the migratory potential of HUCB-MNC fractions. SDF-1 affects
lymphocytes, monocytes (Bleul et al., 1996) and, CD34+ progenitor cells (Aiuti et al.,
1997) expressing the SDF-1 receptor CXCR4. T cells, the most prominent cell type in
our HUCB-MNC preparations, were also the most abundant cells migrating in all
settings (Fig. 2H). But in relation to the total numbers of migrated cells the proportion
of T cells was not noticeably enhanced after OGD (Fig. 2I). Applying control or OGD
supernatants did not influence the proportion of most cell types. Remarkably, only the
proportion of CD34+ cells was enhanced in the OGD system indicating a specific
attraction of stem and progenitor cells to ischemic damaged neuronal tissue. This
homing like process underlines observations describing graft enrichment at lesioned
sites made in animal models of stroke (Taguchi et al., 2004; Piao et al., 2009). In
contrast to the microscopic investigation, where we detected cells preferentially
invading OGD slices, in this experimental set up OGD and control slice homogenates
attracted the same amounts of HUCB-MNC (Fig. 2G). Most probably, additional
migration enhancing molecules released during homogenisation does account for
this result.
### 8.5.4 Modulation of NGF secretion by HUCB-MNC

Previous results obtained in the neuroblastoma cell culture model showed a markedly increase in neuronal VEGF secretion. This hypoxia induced VEGF production was reduced after direct co-cultivation of neuronal cells with HUCB-MNC (Reich et al., 2008). Based on those previous observations we monitored VEGF in the slices to learn more about the neuronal response to OGD and HUCB-MNC application. In contrast, HUCB-MNC application after OGD did not reduce VEGF secretion by hippocampal cells (Fig. 4). Explanations for this observation could be glial influences on neuronal VEGF secretion in the organotypic system or a different regulation in the release of VEGF from hippocampal glial cells (Schmid-Brunclik et al., 2008). This result again emphasizes the specific scope of different culture systems.

NGF has been shown to reduce neuronal death in the gerbil hippocampus after ischemia (Shigeno et al., 1991). Different from VEGF, secretion of rat NGF was decreased after direct or indirect HUCB-MNC administration (Fig. 4). Reduction of neuronal damage after HUCB-MNC application could be associated with increased preservation of NGF receptive structures causing the observed decrease of unbound NGF in supernatants. More probably NGF reduction was due to decreased secretion by glial cells shown to upregulate NGF expression after ischemia induced neuronal damage (Himeda et al., 2007). In this case observed NGF reduction would account for reduced neuronal damage and subsequently diminished glial activation after HUCB-MNC application.
8.6 Conclusions

In summary, the experiments described in this study indicate that HUCB-MNC (i) can enhance survival rates of pyramidal cells in hippocampal slices damaged by OGD and (ii) do promote neuroprotection at least in part not by direct cellular contact to injured neurons. Furthermore, CD34\(^+\) cells, a fraction of HUCB-MNC, were shown to preferentially provide neuroprotection in early stages following ischemia and are able to specifically accumulate at damaged tissue sites.
8.7 Abbreviations

APC, allophycocyanin; BDNF, brain derived neurotrophic factor; CTNF, ciliary neurotrophic factor; CTO, Celltracker Orange™; EGF, epidermal growth factor; eYFP, enhanced Yellow Fluorescent Protein; FITC, fluorescein isothiocyanate; HUCB, human umbilical cord blood; IGF-1, insulin-like growth factor 1; IL, interleukin; MNC, mononuclear cells; NGF, nerve growth factor; OGD, oxygen glucose deprivation; PBS, phosphate buffered saline; PE, phycoerythrin; PE-Cy7 phycoerythrin-cyanin7; PI, propidium iodide; VEGF vascular endothelial growth factor.
8.8 Supplementary Material

**Figure S1**

Supp. Fig.:1+2: Chronological sequence of experimental setup

OGD was performed on 12 days old hippocampal slices. One day before the OGD procedure predamaged slices were excluded from the experiment by using PI (Supp. Fig.1). Subsequent to 40 minutes OGD cells (MNC, CD34+, CD34- or Jurkat T cells) were directly administrated onto the slices (Supp. Fig.1, Supp. Fig.2). Alternatively MNC were applied into the culture medium (Supp. Fig.1, Supp. Fig.2). All co-cultures were analyzed on three consecutive days.

Abbreviations in Supp. Fig.2: a cell culture dish, b OHC, c cell culture insert, d culture media, e pipette applying cells directly onto OHC, f pipette applying cells into the culture media.
9 Summary

As a compilation of two publications and unpublished material this work addresses the question which fraction (HUCB-MNC, CD45+/CD34+, and CD45+/CD133+) of the heterogeneous HUCB is most sufficient in providing neuronal protection after experimental stroke and what are mechanisms of action. A post hypoxic cell culture model and hippocampal slice cultures subjected to OGD were utilized to investigate molecular mechanisms involved in the interplay between HUCB fractions and damaged neuronal cells or tissue. HUCB fractions under investigation were directly or indirectly administered and their effect was investigated through three consecutive days. The molecular micro environment created by hypoxically injured neuronal cells stimulated all fractions of HUCB to secrete neurotrophic factors and/or immunologically active substances influencing neuronal apoptosis.

HUCB-MNC showed a compelling neuroprotective capacity in direct as well as in indirect co-cultures. Directly administered HUCB-MNC drastically reduced rates of neuronal apoptosis to range of controls. That might be caused through a marked increase of chemokines CCL5; CCL3 and CXCL10 released by HUCB-MNC which after active migration formed clusters in direct cell-cell contacts with axons and somata of neuronal cells. Surprisingly CD45+/CD133- had comparable potential to HUCB-MNC. Within indirect co-cultures this stem cell reduced fraction produced high concentrations of CCL3 and neuroprotective G-CSF triggering the retaining of neuronal phenotypes.

CD45+/CD133+ stem cells separated from HUCB-MNC significantly reduced neuronal apoptosis in direct co-cultivations; however, soluble factors produced by CD45+/CD133+ cells were not detectable in a measurable concentration.

Results obtained from hippocampal slices subjected to ODG showed that HUCB-MNC can mediate direct neuroprotection especially when they were directly administered in sufficient cell numbers (12.5x10^4 MNC per slice). In addition, CD45+/CD34+, but not CD45+/CD34- cell fractions separated from HUCB-MNC transiently reduced the number of degenerating pyramidal neurons by a reduced secretion of nerve growth factor.

Results obtained from the two different in vitro models that were employed here indicate that neuroprotection by HUCB-MNC is a stable approach to provide
neuroprotection while the divergent results for stem cell fractions differ and indicate a strong system dependency. Particularly in regard to the clinical use there seems to be no benefit in the use of pure stem cell fractions gained from HUCB-MNC that justifies the effort on selection of that small amount of cells from the HUCB-MNC fraction.
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11 Declaration of Authorship

I hereby declare that the present work was prepared independently, without help from others, and without using anything other than the named sources and aids. I confirm that others did not either directly or indirectly receive any payment in kind for any work related to the content of this dissertation. Neither in Germany nor abroad, the same or a similar work, has been submitted to any other examination or at any other educational institution for the purpose of a dissertation. Where I have consulted the published work of others, this is always clearly attributed. All persons directly involved in the development of this work are named.

Signed: ................................................

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Evidence for neuroprotective properties of human umbilical cord blood cells after neuronal hypoxia in vitro

Susann Hau†1, Doreen M Reich*†1, Markus Scholz4, Wilfried Naumann3, Frank Emmrich1,2,5, Manja Kamprad†2,5 and Johannes Boltze†1,5

Address: 1Fraunhofer-Institute for Cell Therapy and Immunology, Deutscher Platz 5e, 04103 Leipzig, Germany, 2University of Leipzig, Institute of Clinical Immunology and Transfusion Medicine, Johannisallee 30, 04103 Leipzig, Germany, 3University of Leipzig, Faculty of Biology, Pharmacy and Psychology, Institute of Zoology II, Talstrasse 33, 04103 Leipzig, Germany, 4University of Leipzig, Institute of Medical Informatics, Statistics and Epidemiology, Haertelstrasse 16-18, 04107 Leipzig, Germany and 5Translational Centre for Regenerative Medicine, Philipp-Rosenthal-Strasse 55, 04103 Leipzig, Germany

Email: Susann Hau - susann.hau@izi.fraunhofer.de; Doreen M Reich* - doreen.reich@izi.fraunhofer.de; Markus Scholz - markus.scholz@imise.uni-leipzig.de; Wilfried Naumann - naumann@rz.uni-leipzig.de; Frank Emmrich - frank.emmrich@izi.fraunhofer.de; Manja Kamprad - kamm@medizin.uni-leipzig.de; Johannes Boltze - johannes.boltze@izi.fraunhofer.de

* Corresponding author    †Equal contributors

Abstract

Background: One of the most promising options for treatment of stroke using adult stem cells are human umbilical cord blood (HUCB) cells that were already approved for therapeutic efficacy in vivo. However, complexity of animal models has thus far limited the understanding of beneficial cellular mechanisms. To address the influence of HUCB cells on neuronal tissue after stroke we established and employed a human in vitro model of neuronal hypoxia using fully differentiated vulnerable SH-SY5Y cells. These cells were incubated under an oxygen-reduced atmosphere (O2< 1%) for 48 hours. Subsequently, HUCB mononuclear cells (MNC) were added to post-hypoxic neuronal cultures. These cultures were characterized regarding to the development of apoptosis and necrosis over three days. Based on this we investigated the therapeutic influence of HUCB MNC on the progression of apoptotic cell death. The impact of HUCB cells and hypoxia on secretion of neuroprotective and inflammatory cytokines, chemokines and expression of adhesion molecules was proved.

Results: Hypoxic cultivation of neurons initially induced a rate of 26% ± 13% of apoptosis. Hypoxia also caused an enhanced expression of Caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP). Necrosis was only detected in low amounts. Within the next three days rate of apoptosis in untreated hypoxic cultures cumulated to 85% ± 11% (p ≤ 0.001). Specific cytokine (VEGF) patterns also suggest anti-apoptotic strategies of neuronal cells. Remarkably, the administration of MNC showed a noticeable reduction of apoptosis rates to levels of normoxic control cultures (7% ± 3%; p ≤ 0.001). In parallel, clustering of administered MNC next to axons and somata of neuronal cells was observed. Furthermore, MNC caused a pronounced increase of chemokines (CCL5; CCL3 and CXCL10).

Conclusion: We established an in vitro model of neuronal hypoxia that affords the possibility to investigate both, apoptotic neuronal cell death and neuroprotective therapies. Here we employed the therapeutic model to study neuroprotective properties of HUCB cells.

We hypothesize that the neuroprotective effect of MNC was due to anti-apoptotic mechanisms related to direct cell-cell contacts with injured neuronal cells and distinct changes in neuroprotective, inflammatory cytokines as well as to the upregulation of chemokines within the co-cultures.
Background

Acute ischemic stroke is characterised by the immediate depletion of oxygen and glucose in brain tissue. A residual cerebral blood flow (CBF) of $\leq 6 \text{ cm}^3 \times 100 \text{ g}^{-1} \times \text{min}^{-1}$ representing severe ischemia is associated with a nearly total loss of energy on vulnerable neurons. Ischemia therefore rapidly culminates in the formation of a necrotic core [1]. In the penumbra, mild ischemia (CBF $11-20 \text{ cm}^3 \times 100 \text{ g}^{-1} \times \text{min}^{-1}$) leads to the activation of complex neurochemical cascades of cell death, mainly apoptosis. In principle these apoptotic cascades are reversible and form an important aspect of the penumbra concept, which is the major target of therapeutic interventions [2,3]. Recent findings indicate that transplantation of external cell fractions could accompany established therapeutic procedures limited by narrow time windows [4], but the underlying processes are still rather unclear.

Our insights into pathophysiological processes and new therapeutic strategies have mostly been obtained from animal models of focal cerebral ischemia [5,6] and rodent organotypic hippocampal slice cultures [7-9]. However, the complexity of those systems has limited the detailed understanding of mechanisms related to ischemic brain injury [10] and possible interfering effects of cellular therapies [11] so far. Furthermore, results obtained from rodent models are not completely and unobjectionably transferable to human therapy [12,13]. Consequently, experimental expenditure and ethical considerations demand in vitro models representing the main properties of stroke-related processes as neuronal apoptosis to accompany more complex model systems. This would allow to answer explicit questions concerning the role of cell-cell interactions and production of metabolites to verify observations made in in vivo models. It furthermore offers the possibility to precisely manipulate extra cellular environments.

Well described human neuronal cell lines exhibit a multitude of characteristics of typical central-nervous-system (CNS) neurons, overall cell material can be achieved in large quantities. Therefore, human neuronal cell lines, such as the teratocarcinoma NT-2 cell line, became useful tools to study the effects of hypoxic conditions on neurons [14]. However, the utilisation of NT-2 neuronal cultures is restricted by time-consuming and expensive differentiation periods of up to 44–54 days [15,16] that are also sensitive to environmental disturbances. In contrast, the SH-SYSY neuroblastoma cell line was shown to be differentiated into neuronal cells within a comparatively short time of 16 days [17]. Furthermore, the cell line fits major relevant criteria (high vulnerability, irreversible differentiation into pure neuronal cells) to serve as a model of hypoxic injury of central neurons [18]. Hence, our exclusive human model of neuronal hypoxia forms the basis to identify possible anti-apoptotic neuroprotective potentials of therapeutic supplements. Mononuclear cells (MNC) from human umbilical cord blood (HUCB) were shown to improve functional outcome of animals after focal cerebral ischemia. The cellular effects causing the observed benefits are not fully understood for these cells [19,20]. In this context we investigated whether injured post-hypoxic neuronal cells or MNC initiated an anti-apoptotic response mediated by cytokines or chemokines.

Results

After differentiation SH-SYSY exhibited neuronal morphology and specific neuronal markers

Sixteen days of differentiation yielded cultures of fully matured neuronal cells shown in Fig. 1A, B. Following the seventh day of differentiation, the majority of SH-SYSY cells were stained positive for the specific neuronal markers (β-tubulin III, taurin I, neuron-specific enolase [NSE], neurofilament [NF] H/M and microtubule-associated proteins [Map] 2a/b). At Day 16 all cells exhibited all of these markers. Time course of marker expression showing continuously increasing stages of differentiation is given in Fig. 1C. Markers showed typical localisation to cytoplasm and dendrites. The explicit majority of differentiated SH-SYSY cells (73% ± 11%) resembled typical neuronal morphology with round phase-bright somata and long, terminal-branched dendrites forming a dense network (Fig. 1A, arrow 1). However, the neuronal culture is also characterised by a cell type (only 27% ± 11%) which shows abundant cytoplasm and a lack of axonal extensions (Fig. 1A, arrow 2). Remarkably, no differences were noted with regard to expression of neuronal markers. Furthermore, the number of this type of cells remained constant during the culture time.

Absence of proliferation after termination of differentiation

At Day 4 of differentiation the total number of SH-SYSY cells, counted by nuclear staining with DAPI, increased by twofold (21.2 ± 6.7 × 10^3/cm²) since seeding. Beginning on the seventh day of culture numbers of nuclei remained nearly stable (18.2 ± 2.2 × 10^3/cm²). Subsequent to hypoxia, there was no significant alteration in numbers of counted nuclei. Hence, the influence of oxygen deprivation induced no further proliferation and also no significant loss of cells in comparison to normoxic control cultures (Fig. 2). Therefore all following results should be seen in the context of the nearly constant numbers of neuronal cells under normoxic and hypoxic cultivation conditions. It is of note that the amount of stained nuclei as the physiologic status of these cells. On the whole the cultures include cells in a viable, apoptotic or necrotic state.
Hypoxia induced apoptosis in the majority of neuronal cells

Post-hypoxic and normoxic control cultures exhibited pronounced differences in the quantity of apoptotic cells as well as in cell morphology. Hypoxic conditions for 48 hours induced an initial apoptosis rate of 26% ± 13%. There was a continuous increase in the apoptotic cell fraction to 85% ± 11% within 3 days post-hypoxia as compared to control cultures (p ≤ 0.001; Fig. 3). The clearest effects of oxygen deficiency were seen three days following induction of hypoxia as shown by annexin-V staining (green fluorescence, Fig. 4A and 4B). Additionally, post-hypoxic cultures were characterised by retracted dendrites, indicating a loss of multiple cell-cell contacts. Debris and apoptotic bodies were found in most culture dish areas, evidencing late stage of apoptosis (Fig. 4A). In contrast, normoxic cultures displayed a much more reduced amount of apoptotic cells (Fig. 4B). Over the whole time, apoptosis in these control cultures remained stable at about 7% ± 3% (Fig. 3).

After hypoxia, on Day 0 and Day 1, number of late apoptotic/necrotic cells significantly increased up to 27% ± 13%. Further progression of propidium iodide (PI) posi-
tive cells in post-hypoxic cultures resulted in 23 ± 16% on Day 3 (Fig. 5). By comparison, in normoxic control cultures, necrosis levels remained stable below 17% and therefore did not statistically differ from post-hypoxic cultures on this time point. This fact was corroborated by images that show no observable deviation in the amount of necrotic and late apoptotic cells, as indicated by PI staining (red fluorescence, Fig. 4C and 4D).

In general, an increased release of calcein-AM also indicated a strong decrease of neuronal cell viability which verifies the results of the apoptosis and necrosis rates (data not shown).

Hypoxia increased quantities of Caspase-3 and cleaved PARP immediately and secretion of VEGF in delay

Bar charts in Figure 6 show the concentrations of Caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP). In post-hypoxic cultures apoptotic proteins drastically rose after oxygen deprivation. The highest concentration of Caspase-3 was measured directly after hypoxia on Days 0 and 1 (2-fold and 5.8-fold, respectively). From Day 2 on, the level of active Caspase-3 sharply decreased. The distribution of cleaved PARP levels showed patterns similar to Caspase-3. This decline was accompanied by a secretion of VEGF on Day 3 after hypoxia (Fig. 7).

Adhesion molecules (L1, NCAM and ICAM-1) were upregulated after hypoxia

Independent of cultivation conditions immunofluorescence analysis revealed that nearly all (98%) of the cells were positive for neurite cell adhesion molecule (L1; 98.3 ± 0.1%) and Neural Cell Adhesion Molecule (NCAM; 99 ± 0.8%) at Day 0. L1 and NCAM were detected on somata and multiple dendrites. Furthermore, a few of the neuronal cells also expressed Vascular Cell Adhesion Molecule (VCAM-1; 5.7%) and Intercellular Adhesion Molecule (ICAM-1; 14.6%) on their somata and dendrites. There was no change in relative numbers of marked cells and expression patterns in post-hypoxic cultures. To quantify the density of adhesion molecule expression, highly sensitive fluorescence measurement of supernatants of lysed cultures was performed. Here, comparative studies between cultures directly after 48 hours of hypoxia and normoxic control cultures showed a significant upregulation of L1, NCAM and ICAM-1 (Fig. 8). Hypoxic condi-
tions determined a considerable increase in L1 and NCAM protein expressions of up to 189% ± 74% and 155% ± 50%, respectively. Hypoxia also strongly upregulated the ICAM-1 expression up to 424% ± 251%. The level of expression of VCAM-1 was not altered by the hypoxic environment. The continued degradation of the dendritic networks did not allow assaying the specific expression of adhesion molecules at late stages of post-hypoxic cultures.

Co-culturing with HUCB MNC strongly reduced apoptosis in post-hypoxic neuronal cell cultures

We found noticeable neuroprotective properties of HUCB MNC in the co-culture experiments. Untreated post-hypoxic mono-cultures of neuronal cells showed 85% ± 11% of apoptosis after three days, whereas in co-cultures with MNC the rate of apoptosis was stable at a level of 6.3% ± 1% (p ≤ 0.001). This is comparable to normoxic control cultures (7% ± 3%; Fig. 9A). Photographs of co-cultures with MNC revealed clear effort of MNC to localise next to somata and branches of post-hypoxic neuronal cells. In the course of clustering MNC mostly avoided areas that were not settled by neuronal cells (Fig. 9B). Furthermore, the administration of MNC showed positive influence on the conservation of neuronal networks as compared to cultures that did not receive any cell therapy (Fig. 4A and 4C).

MNC increased CCL5, CCL3, CCL4 and CXCL10 in post-hypoxic neuronal cultures

The investigation of neuroprotective cytokines (G-CSF, VEGF) (Fig. 7A), inflammatory cytokines (IL-1β, IL-6, CXCL8) (Fig. 7B) and chemokines (CCL2, CCL5, CCL3, CCL4, CXCL10) (Fig. 7C) in supernatants of normoxic neuronal cultures revealed considerable amounts of CXCL8, CCL2 and VEGF. Remarkably, after three days under hypoxic conditions only VEGF was strongly upregulated (about threefold increase). After administration of MNC, VEGF was downregulated regarding to the total concentration measured in mono-cultures of MNC and in post-hypoxic neuronal cells. Inflammatory cytokines as IL-1β, IL-6 and CXCL8 were produced by MNC. The secretion of cytokines such as IL-1β and IL-6 was not altered in
post-hypoxic co-cultures, whereas CXCL8 was suppressed. Interestingly, the majority of chemokines was clearly upregulated in co-cultures with MNC. We could show that CCL5, CCL3, CCL4 and CXCL10 were increased up to the tenfold (CCL3, CCL4), whereas the concentration CCL2 was not regulated.

**Discussion**

In this study, we introduce an experimental human in vitro model to investigate (i) the mechanisms of neuronal hypoxia and (ii) the interaction of neuronal cells with external stem cell-containing fractions as possible therapeutic tools. According to the differentiation protocol of Encinas et al. [17] we obtained fully matured neuronal cells after 16 days. Previous, orientating experiments showed typical response of differentiated SH-SY5Y cells to N-methyl-D-aspartate (NMDA; 300 μM) application and most pronounced vulnerability after 48 hours hypoxic incubation (data not shown). These post-hypoxic neuronal cultures can be employed already 18 days after the seeding of naive cells. Therefore the time period until the model of hypoxic neuronal cells is available is very short. In comparison to other approaches that provide assimilable hypoxia models using teratocarcinoma cell lines [16] the generation of our model is shortened by at least 10 days [21]. The proved expression of specific neuronal markers and the absence of any proliferation gave clear evidence of matured neuronal cells in the G0-phase of the cell cycle. This steady state allows making direct conclusions about the influence of any external manipulation because any change will most probably be due to these procedures. Hence, the model of neuronal hypoxia

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**Figure 4**

**Long term effect of 48 hours of hypoxia on apoptosis (A-B) and necrosis (C-D).** Cultures at Day 3 after hypoxia were compared with normoxic cultures of the same age. Combined phase contrast/fluorescence micrographs of neuronal post-hypoxic cultures (A) and normoxic control cultures (B) show a conspicuous increase of apoptotic cells (annexin-V-staining, green fluorescence) and morphologic changes due to hypoxia (cell debris, retraction of dendrites). Propidium iodide (PI, red fluorescence) staining shows the influence of hypoxia (C) on the number of necrotic cells and cells in a late state of apoptosis compared to control cultures (D). In contrast to the level of apoptosis, there was no clear difference in the number necrotic cells following both culture conditions.
affords the possibility to investigate manifold questions concerning mechanisms triggered in response to hypoxia and therapeutic interventions e.g. application of HUCB MNC.

Following hypoxia, neuronal cultures displayed a correlation between morphological changes and increase of annexin-V positive cells as well as changes in adhesion molecule expression. The specific apoptosis marker annexin-V indicated that hypoxic cultivation preferentially induced apoptosis. Moreover, there was a continuous enlargement in number of apoptotic cells from initial 26% ± 13% until cultures almost completely consisted of apoptotic cells (85% ± 11%) within three days. Conforming to changes of morphology in post-hypoxic cultures, there was an enhanced release of calcein-AM also observable due to the loss of the integrity of membrane. This is typical for late stages of apoptosis as well as of necrosis. The model of neuronal hypoxia was approved by moderate rates of PI positive cells (23% ± 16% at Day 3 post-hypoxia). Therefore the overrun of 100% by the summation of annexin-V positive and PI positive cells is due to a proportion of cells in a late state of apoptosis that are positive for annexin-V and PI [22]. We focused on apoptosis because it is a reversible process which could be modulate by external stimulation [23,24]. Moreover after stroke cell death in the penumbra is predominantly considered to be apoptosis [25-28]. Therefore apoptosis is a therapeutic target of anti-apoptotic therapies like stem cells or cytokines [29-32].

Apoptosis in the penumbra takes place in a time span of about 72 hours after vessel occlusion in the rat after middle cerebral artery occlusion (MCAO) [33]. This time course is represented by the model system introduced in this study. The fact that there was no major alteration in the number in DAPI-positive nuclei underlines that the influence of hypoxia did not lead to an immediate destruction of the cells but allows investigating apoptosis and subsequent therapeutic interventions within 72
hours. These facts were also confirmed by characteristic changes in the morphology of post-hypoxic cells. There was a total retraction of dendrites and a resulting destruction of neuronal networks, grained cell surfaces and extended cell degradation. This damage was accompanied by an upregulation of neuronal adhesion molecules. We hypothesize that this upregulation indicates a cellular answer to compensate for a loss of direct cell-cell interaction as a consequence of the preceding hypoxic stress as described by several authors [34-36]. However, the final loss of intercellular networks could not be compensated by the increased expression of neuronal adhesion molecules alone.

Neuronal cell death after hypoxia is caused by membrane depolarisation subsequent to energy failure [37,38]. The resulting calcium overload is the initial point of the activation of manifold biochemical pathways that affect caspases, free radicals and gene expression [39,40]. The specific apoptotic proteins Caspase-3 and cleaved PARP were quantified to prove whether our microscopic observations are corroborated by biochemical pathways [41-43]. After 24 hours post-hypoxia, both marker proteins extensively increased. This was followed by a sharp drop in their expression level. This reduced amount of cleaved PARP might be due to an aggravating energy failure in the cell. This in turn is attributed to the high levels of cleaved PARP, evidenced above, which caused adenosintriphosphate (ATP) depletion [44,45] and which in this context prevents the enzymatic activity of caspases. Furthermore, an increase of the expression of VEGF, a key mediator of angiogenesis [46,47], was observed in higher concentrations in late post-hypoxic cultures. This might be a reflection of compensatory in vivo processes of revascularisation in our model [48,49]. VEGF is also known to be involved in neuronal protection through inhibition of Caspase-3 [50,51]. The time-delayed increased release of VEGF as an effect of hypoxia does not seem to influence the number of apoptotic cells in our cultures. This may be a result of concentrations of VEGF that did not reach efficient thresholds. In this context the measured reduction of caspases on Days 2 and 3 after hypoxia is caused by the already described ongoing energy failure in the post-hypoxic cells.

Figure 6
Effect of 48 hours of hypoxia on the concentration of active Caspase-3 and cleaved PARP. Data are taken in a time course of three days after hypoxia and are based on pooled lysates of the totality of SH-SYSY cells taken out of 12 individual wells. Data are expressed in units of protein [active Caspase-3] and cleaved protein [PARP] per millilitres. Hypoxia induced apoptosis specific proteins (Caspase-3, cleaved PARP) in a time-related manner.
However, the regulation of those apoptotic proteins is probably not related to VEGF mediated-effects.

The therapeutic benefit of cell administration after stroke has been demonstrated in numerous animal studies [52,53]. However, important questions in basic cellular and molecular mechanisms of neuronal cell death and its prevention after inadequate oxygen supply still remain unanswered. To understand the mechanisms of cellular neuroprotection after stroke we employed the model of neuronal hypoxia and applied MNC from HUCB.

In vivo MNC are supposed to differentiate into neuronal cells [54,55] to trophically support neuronal tissue through the production of growth factors [56], to support the new formation of synapses and migration as well as the differentiation of endogenous neuronal progenitors [54,55]. In our experiments, the administration of MNC from HUCB to post-hypoxic neuronal cultures showed remarkable beneficial effects. Over three days we could show a clear reduction of neuronal apoptosis, even to the level of normoxic control cultures ($7\% \pm 3\%$). There are two observations that contribute to an explanation of neuroprotective mechanisms. First, MNC were preferentially located close to hypoxically injured neuronal cells. This phenomenon was facilitated through an intensive upregulation of ICAM-1 on neuronal cells which is the specific ligand for leukocyte cell adhesion molecule [LFA-1].

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**Figure 7**

Cytokine profile in hypoxic injured SH-SY5Y cells and in direct co-cultures with MNC at Day 3. Cytokine concentrations were measured in supernantes via cytometric bead array. Data originate from five independent experiments and are expressed as ng/mg. "+" indicates significant differences in cytokine concentration of post-hypoxic SH-SY5Y mono-cultures compared to normoxic controls. Double bars are the sum of cytokine concentration from mono-cultures. "*" shows significant differences in cytokine concentration in co-cultures with MNC compared to the total of mono-cultures. Note different scaling.
1] expressed by all immune cell subsets [57]. However, the formation of cell chimera was not observed, as there were no CFSE-stained cells with neuronal morphology. In future experiments we will investigate whether blocking of adhesion molecules on the surface of neuronal cells can inhibit the co-localisation of MNC. Further it will be proved whether this influences the rate of apoptosis. An increase of apoptosis would be an evidence for the significance of direct cell-cell-contacts in context of therapeutic mechanisms of MNC. The second observation would be concomitance of an MNC indicated specific alteration in levels of soluble factors. That change might be held responsible for neuroprotection. Pronounced upregulation of chemokines (CCL5, CCL3, CCL4, CXCL10) might be causal for the enhanced effort of MNC to localise near neuronal structures. High concentrations of VEGF are known to be neuroprotective [58,59]. In contrast to post-hypoxic mono-cultures, we found no increase of VEGF after administration of MNC. It seems that presence of MNC inhibited the induction of elevated levels of VEGF and compensated its neuroprotective effects by other mechanisms. Cord blood contains distinct cell types capable to differentiate into neuronal cells [60]. However, the differentiation of stem cells (about 1% within the MNC fraction) was not expected because of the short time of co-cultivation that clearly undershoots time frames responsible for neuronal differentiation.

So far, it is unclear whether neuroprotection resulted from one of the observed effects or from a combination of spatial proximity and specific cytokine patterns. Consequently, further studies in indirect co-cultures will be necessary.

**Conclusion**

Pathophysiological models developed from animal studies form the basis of our understanding of the development of stroke. *In vivo* data display a perfusion-related dependency of neuronal cell damage. Residual energy supply in the penumbra induces apoptosis, the early phases of which are reversible. Consequently, rescue of the penumbra is a major target of experimental stroke therapy. Data obtained from complex animal models of stroke strongly suggest that transplanted cells enhance neuronal survival.

We established a standardized human *in vitro* model of neuronal apoptotic cell death after hypoxia that can facilitate to address specific pathophysiological processes underlying hypoxic damage and cell-mediated neuroprotection more precisely.
Interestingly, transplanted MNC not only strongly decreased the ratio of apoptosis in neuronal cells but also triggered retaining neuronal characteristics such as forming networks. MNC clustered around post-hypoxic neuronal cells and induced an alteration in cytokine and chemokine concentrations. Our data suggest that the neuroprotective effects of MNC might result from direct cell-cell contacts and/or the adjustment of specific soluble mediators.

**Methods**

**Cultivation and differentiation of neuronal cells**

All experiments were performed using SH-SY5Y human neuroblastoma cells (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) between passages 4–7. The common medium was Dulbecco's Modified Eagle Medium (DMEM, high glucose 4.5 g/l, L-Glutamine 580 mg/l; PAA, Pasching, Austria) with penicillin G (10,000 U/ml; PAA Laboratories, Pasching, Austria) and streptomycin (10 mg/ml; PAA Laboratories, Pasching, Austria). Cells were maintained in MM in a humidified atmosphere with 5.5% CO₂ at 37°C (Table 1). When cultures achieved subconfluence, cells were subcultured with trypsin/EDTA (PAA Laboratories, Pasching, Austria). Differentiation was carried out according to the protocol of Encinas and colleagues [17] but was adapted as follows: cells were plated at an initial density of 0.9 × 10⁴/cm² in 16-mm-diameter cavity (Greiner Bio-One, Frickenhausen, Germany) and differentiated over a period of 16 days in relevant media and cultured thereafter according to Table 1. Cultivation procedure is illustrated by Fig. 10.

**Immunocytofluorescence of neuronal markers**

The primary antibodies used against neuronal epitopes were: β-tubulin III (rabbit 10 μg/ml; BD PharMingen, Heidelberg, Germany), taurin I and neurofilament (NF) H/M (rabbit 5 μg/ml and mouse 1:200; Chemicon, Hampshire, UK), neuron-specific enolase (NSE) and microtubule-associated proteins (Map) 2a/b (mouse 1:2 and mouse 5 μg/ml; Sigma-Aldrich, Munich, Germany). Fluorochrome-conjugated secondary antibodies were purchased from DAKO, Carpinteria, CA, USA (goat anti-mouse-PE [1:200] and pork anti-rabbit-FITC [1:30]). Indirect immunostaining was processed according to manufacturer's instructions.

**Hypoxia and post-hypoxic cultivation**

Before enddifferentiated neuronal cells were exposed to hypoxia they were refreshed with DM. Hypoxic condi-

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**Figure 9**

**Effect of co-culturing of MNC on ratio of neuronal apoptosis and preservation of neuronal networks.** Apoptosis was induced by 48 hours incubation of neuronal cells under hypoxic conditions. Afterwards 4.5 × 10⁵ CFSE stained MNC were directly applied to neuronal cells (0.3 × 10⁵/well). For three days co-cultures were observed under normoxia. In co-cultures with MNC rate of apoptosis was clearly reduced compared to post-hypoxic cultures (A). Combined phase contrast and fluorescence micrograph of post-hypoxic neuronal cells and MNC (green) in direct co-culture (B).
tions were $O_2 < 1\%$ (oxygen substituted with nitrogen) and lasted 48 hours in a 37°C tempered and humidified incubator (Binder GmbH, Tuttlingen). After hypoxia, cultures were supplied with PHM (Table 1) and were transferred back to normoxic conditions for the following three days (Fig. 10).

**Quantification of cell numbers by nuclear staining**

The total number of cells was measured by nuclear staining with 4’, 6-diamidino-2-phenylindole (DAPI, Invitrogen, Karlsruhe, Germany). Cells were washed and stained with 1 $\mu$g/ml (DAPI/Methanol) for 15 minutes at 37°C. 25 microphotographs of randomised fields were taken per cavity using a Zeiss fluorescence microscope (Carl Zeiss AG, Jena, Germany) equipped with Zeiss AxioVision-Software. The number of nuclei was automatically determined by means of Zeiss AutMess (Carl Zeiss AG, Jena, Germany).

**Cell viability assay**

Apoptosis was determined via annexin-fluorescein isothiocyanate (FITC) or annexin-phycocerythrin (PE; both 1:20 reaction buffer; BD PharMingen, Heidelberg, Germany) using fluorescence microscopy. Necrosis was identified by propidium iodide (PI) staining (1 $\mu$g/ml phosphate-buffered saline [PBS]; Bender medSystems, Vienna, Austria). Both methods detect cells in a late state of apoptosis. Apoptosis and necrosis were ascertained in different cavities. Calcein-AM (40 $\mu$M/PBS; Invitrogen, Karlsruhe, Germany) was utilised for detection of living cells.

**Quantification of apoptotic proteins**

BD Cytometric Bead Array for human apoptosis (Becton Dickinson, Erembodegem, Belgium) was used for the quantitative measurement of apoptotic proteins (cleaved PARP and Caspase-3). Manufacturer’s instructions were adapted to lyse cells directly within multi-well plates. For cell lyses differentiated cells were rinsed with PBS and incubated on ice in the provided buffer for 20 minutes.

**Determination of adhesion molecules by immunocytochemistry and cell-based fluorescence measurement**

The cultures were stained with antibodies against CD56-phycocerythrin (PE; NCAM), CD171 (L1, both Becton Dickinson, Erembodegem, Belgium), CD54-PE (ICAM-1; Immunotech, Hamburg, Germany) and CD106-PE (VCAM-1; Southern Biotech, Birmingham, Alabama, USA) in order to investigate the distribution and localisation of adhesion molecules on differentiated neuronal cells. The staining protocol proceeded as follows: Medium (DM, Table 1) was removed and dishes were washed twice with PBS. The cells were incubated with primary labelled or unlabelled antibodies (1:100) for 10 minutes at 37°C and afterwards washed in PBS. Thereafter the cells were incubated with goat anti-mouse-PE (DAKO, Hamburg.

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**Figure 10**

Schematic illustration of experimental set up. Sixteen days of differentiation were followed by 48 hours cultivation under hypoxic conditions. Following hypoxia cultures were maintained under normoxic conditions for three days and are referred as post-hypoxic. Subsequently, cultures were analysed daily.
Germany) antibody for 10 minutes at 37°C for CD171 detection. After labelling the cultures were immersed in PBS.

The spatial distribution of adhesion molecules was observed using fluorescence microscopy (Carl Zeiss AG, Jena, Germany). The density of molecule expression was measured in cell lysates. Therefore, antibody-labelled cultures were treated with 1% Triton × 100 (Ferak, Berlin, Germany)/PBS at 37°C. Whole-cell lysates were transferred to black 96-well-plates (Greiner Bio-One, Frickenhausen, Germany). PE-fluorescence signals were determined with a spectrafluorometer (Tecan Spectrafluor Plus, Tecan Trading AG, Switzerland) at an excitation wavelength of 488 nm and an emission wavelength of 590 nm. Specific isotypes and 1% Triton × 100 served as negative controls.

Preparation of Human Umbilical Cord Blood (HUCB) samples

Cord blood samples were obtained anonymously in accordance to ethical prescripts immediately after delivery. HUCB samples of healthy full-term neonates were processed according experienced methods including density gradient separation using Lymphocyte Separation Medium (PAA Laboratories, Cölbe, Germany). Gained MNC fraction was stored by freezing in the gaseous phase of liquid nitrogen after the addition of FCS/8% dimethyl sulfoxide (Serumwerke Bernburg Inc., Bernburg, Germany). Prior to use cryopreserved MNC were thawed rapidly in 75 U/ml DNaseI/0.5 M MgCl₂ (Roche Diagnostics GmbH, Mannheim, Germany/Sigma, Germany) and washed in RPMI (PAA Laboratories, Austria). Cell suspension was stained with carboxy fluoresceindiacetate succinimidyl ester (CFSE 5 μM; Molecular Probes, Inc., Eugene, OR, USA) for 10 minutes at 37°C.

### Co-culture of neuronal cells and MNC

Direct co-culturing of fully differentiated neuronal cells and MNC was carried out under normoxic conditions (37°C) over a period of 3 days following 48 hours of hypoxia. A total amount of 4.5 × 10⁵ CFSE stained MNC were dissolved in 500 μl PHM and were added to the post-hypoxic neuronal cells (0.3 × 10⁵/cavity). The ratio of neuronal cells to MNC was 1:15.

### Cytokine profiling

The supernatants from normoxic cultures on Day 21 as well as from hypoxic cultures on Day 3 post-hypoxia were collected in order to characterise soluble factors produced by cultured cells. They were detected simultaneously by means of Becton Dickinson Cytometric Bead Array. Supernatants were tested for neuroprotective (Granulocyte Colony-Stimulating Factor [G-CSF], Vascular Endothelial Growth Factor [VEGF]) and inflammatory cytokines (Interleukin [IL]-1β, IL-6, CXCL8) as well as chemokines (CXCL10, CCL3, CCL4, CCL5, CCL2). The detection limit was 0.02 ng/ml, except for VEGF and CCL2 (0.04 ng/ml).

### Statistical analyses of data

Except for apoptosis and necrosis rates all results have been reported as mean values ± SD. Statistical differences were analysed by the Student s-t-test or the Mann-Whitney rank sum-test. P values of ≤ 0.05 were considered statistically significant (* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001). Apoptosis and necrosis rates were logit-transformed to obtain normally distributed quantities. The effects of time, experimental setting (hypoxia), experimental run and the investigated cavity were determined univariately, and, finally multivariately using a mixed-model approach with time and experimental setting as fixed effects and cavity and experimental run as random effects. Cytokine concentrations were compared between the normoxic and the hypoxic group by means of the Mann-Whitney rank

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### Table 1: Composition of culture media

<table>
<thead>
<tr>
<th>Day of culture</th>
<th>Culture condition</th>
<th>Description of media</th>
<th>FCS (15%)</th>
<th>RA (10 μM)</th>
<th>BDNF (5 ng/ml)</th>
<th>HSA (0.1%)</th>
<th>Ham’s F12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeding</td>
<td>Normoxia</td>
<td>Maintenance medium</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 – 4</td>
<td>Normoxia</td>
<td>Basic medium (BM)</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 – 16</td>
<td>Normoxia</td>
<td>Differentiation medium (DM)</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>16 – 18</td>
<td>Normoxia/hypoxia</td>
<td>Differentiation medium (DM)</td>
<td>-</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>18 – 21</td>
<td>Normoxia</td>
<td>Post-hypoxic medium (PHM)</td>
<td>-</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

The experimental design demanded varying culture media. Supplements in DMEM were purchased from: fetal calf serum (FCS, PAN-Biotech, Aidenbach, Germany), all-trans-retinoic acid (RA, Sigma-Aldrich, Steinheim, Germany), recombinant brain-derived neurotrophic factor (rhBDNF; ImmunoTools, Friesoythe, Germany), human serum albumin (HSA, PAN Biotech, Aidenbach, Germany).
sum test. Cytokine concentrations measured in co-cultures were compared with the sum of the concentrations obtained in the post-hypoxic neuronal cultures and in the MNC mono-cultures via a bootstrapping algorithm. This was performed by resampling and by the addition of concentrations of cytokines measured in post-hypoxic neuronal cultures and MNC mono-cultures. Results were compared with the Mann-Whitney rank sum test. The mean and standard deviations of the sum of the concentrations were also determined by bootstrapping. Bootstrapping analysis was performed using the statistical software package "R" [61,62]. Mixed-model analyses were performed using PROC MIXED from the statistical software package SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Box plots (if applicable) and univariate analyses were determined using the software package SPSS (SPSS Inc., Chicago IL, USA).

**Authors' contributions**

SH, DMR, MK and JB designed and coordinated the study. SH and DMR conducted all experimental work and wrote the manuscript. SH, DMR and MK analysed and interpreted the data. MS performed the statistical analysis. WN and FE critically revised this study. All authors read and approved the final version of the manuscript.

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**References**


60. The R Project for Statistical Computing [http://www.r-project.org/]

Neuronal hypoxia in vitro: Investigation of therapeutic principles of HUCB-MNC and CD133\(^+\) stem cells

Doreen M Reich\(^*\)1, Susann Hau1, Tobias Stahl2, Markus Scholz3, Wilfried Naumann4, Frank Emmrich1,5,6, Johannes Boltze\(^†\)1,5 and Manja Kamprad\(^†\)6

Address: 1Fraunhofer-Institute for Cell Therapy and Immunology, Perlickstraße 1, 04103 Leipzig, Germany, 2University of Leipzig, Faculty of Veterinary Medicine, Department of Anatomy, Histology and Embryology, An den Tierkliniken 43, 04103 Leipzig, Germany, 3University of Leipzig, Institute of Medical Informatics, Statistics and Epidemiology, Haertelstrasse 16-18, 04107 Leipzig, Germany, 4University of Leipzig, Faculty of Biosciences, Pharmacy and Psychology, Institute of Biology II, Talstrasse 33, 04103 Leipzig, Germany, 5Translational Centre for Regenerative Medicine, Philipp-Rosenthal-Strasse 55, 04103 Leipzig, Germany and 6University of Leipzig, Institute of Clinical Immunology and Transfusion Medicine, Johannisallee 30, 04103 Leipzig, Germany

Email: Doreen M Reich\(^*\) - doreen.reich@izi.fraunhofer.de; Susann Hau - susann.hau@izi.fraunhofer.de; Tobias Stahl - stahl@vetmed.uni-leipzig.de; Markus Scholz - markus.scholz@imise.uni-leipzig.de; Wilfried Naumann - naumann@rz.uni-leipzig.de; Frank Emmrich - frank.emmrich@izi.fraunhofer.de; Johannes Boltze - johannes.boltze@izi.fraunhofer.de; Manja Kamprad - manja.kamprad@medizin.uni-leipzig.de

\(^*\) Corresponding author  \(^†\)Equal contributors

Abstract

**Background:** The therapeutic capacity of human umbilical cord blood mononuclear cells (HUCB-MNC) and stem cells derived thereof is documented in animal models of focal cerebral ischemia, while mechanisms behind the reduction of lesion size and the observed improvement of behavioral skills still remain poorly understood.

**Methods:** A human in vitro model of neuronal hypoxia was used to address the impact of total HUCB-MNC (tMNC), a stem cell enriched fraction (CD133\(^+\), 97.38% CD133-positive cells) and a stem cell depleted fraction (CD133\(^-\), 0.06% CD133-positive cells) of HUCB-MNC by either direct or indirect co-cultivation with post-hypoxic neuronal cells (differentiated SH-SY5Y). Over three days, development of apoptosis and necrosis of neuronal cells, chemotaxis of MNC and production of chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9) and growth factors (G-CSF, GM-CSF, VEGF, bFGF) were analyzed using fluorescence microscopy, FACS and cytometric bead array.

**Results:** tMNC, CD133\(^+\) and surprisingly CD133\(^-\) reduced neuronal apoptosis in direct co-cultivations significantly to levels in the range of normoxic controls (7% ± 3%). Untreated post-hypoxic control cultures showed apoptosis rates of 85% ± 11%. tMNC actively migrated towards injured neuronal cells. Both co-cultivation types using tMNC or CD133\(^-\) reduced apoptosis comparably. CD133\(^-\) produced high concentrations of CCL3 and neuroprotective G-CSF within indirect co-cultures. Soluble factors produced by CD133\(^+\) cells were not detectable in direct co-cultures.

**Conclusion:** Our data show that heterogeneous tMNC and even CD133-depleted fractions have the capability not only to reduce apoptosis in neuronal cells but also to trigger the retaining of neuronal phenotypes.

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Background

Transplantation of adult stem cells has been shown to be an auspicious and effective treatment for degenerative and traumatic neurological diseases [1]. Among degenerative neurological disorders acute ischemic stroke is the leading cause of disability and death in industrial nations [2-4]. Acute stroke leads to an increased release of hematopoietic stem and progenitor cells from bone marrow into peripheral blood [5]. It is assumed that these cells take part in self-healing processes occurring after neuronal injury. They are supposed to promote the survival of the injured brain tissue by producing neurotrophic factors [6], to enhance endogenous angiogenesis [7] and neurogenesis [8] or even to transdifferentiate into neuronal cells [9]. However, the stroke induced endogenous release of hematopoietic stem and progenitor cells seems not to be sufficient to compensate massive loss of brain tissue after extended ischemic stroke. Therefore, external application of hematopoietic stem and progenitor cells is expected to complement current treatment of acute stroke based on thrombolytic therapy. An appropriate source of hematopoietic stem cells is the mononuclear cell (MNC) fraction of human umbilical cord blood (HUCB) [10-12]. Transplantation of HUCB-MNC as well as enriched HUCB hematopoietic stem cells into animals which were subjected to focal stroke caused by middle cerebral artery occlusion (MCAO) ameliorated the animals' functional outcome and reduced the lesion size [13]. However, there are still manifold unanswered questions addressing the beneficial influence of such grafts on injured neuronal cells.

It has been documented that there is no neuronal transdifferentiation of hematopoietic stem cells in vitro [14-16]. Though so far there is no convincing proof that locally administered hematopoietic stem cells transdifferentiate into functionally neuronal cells forming the basis of the animals' behavioral progression [17].

It has recently been shown that there is no need for MNC to enter the brain for neuroprotection. Soluble factors like GDNF, NGF, BDNF or G-CSF are known to promote neuroprotection over long-distances [18,19]. This raises many questions about the cellular mechanisms causing the functional improvement after grafting [20]. Prevention of neurons from apoptotic cell death [21] is considered to be supported by the transplantation and could be directly connected to improved tissue conservation, lesion size reduction and superior functional outcome [22].

Cell culture models of neuronal hypoxia complement the exploration of particular interactions between grafts and neuronal tissue. Our study is based on a well established post-hypoxic neuronal cell culture model (SH-SY5Y). This model was used to address (i) the neuroprotective potential of stem cell enriched and -depleted HUCB derived cell fractions, (ii) the impact of these cells especially on apoptotic status of oxygen-deprived neurons, and (iii) the mediation of cell-derived survival signals (soluble or cell-attached).

Results

Direct co-cultivation with each fraction of HUCB-MNC reduced apoptosis in post-hypoxic neuronal cells

Hypoxic cultivation (48 hours) of fully matured neuronal SH-SY5Y cells resulted in an initial rate of apoptosis of 26% ± 13%. Within the following three days rate of apoptosis increased to 85% ± 11%. By contrast, normoxic control cultures showed a stable amount of apoptotic cells (7% ± 3%) over the whole observation time (data not shown). Direct co-cultivation with tMNC and CD133- showed pronounced reduction of neuronal apoptosis. Similar results were obtained after application of CD133+. By application of $4.5 \times 10^3$ CD133+ they were given in equal amounts as they exist in tMNC. Though the whole cell amount of CD133+ was 100 times less than tMNC administered (Fig. 1A).

Levels of necrosis in post-hypoxic control cultures remained nearly stable (approximately 25%) over three days. tMNC and CD133- cell application also induced a significant reduction of necrosis. CD133+ cells did not influence the level of necrosis (Fig. 1B).

In indirect co-cultures tMNC and CD133- were also sufficient to decrease apoptosis of post-hypoxic neuronal cells

Over the entire observation period, direct as well as indirect co-cultivation with tMNC or CD133- exhibited a significant reduction of apoptosis. In all co-culture set-ups percentage of annexin-V positive cells was significantly lower ($p \leq 0.001$) than in post-hypoxic control cultures (Fig. 2A, B). Direct and indirect co-cultivation of CD133- resulted in similar rates of apoptosis continuously below 5% of annexin-V positive cells (Fig. 2B). However, still generating strong neuroprotective effects, the number of apoptotic neuronal cells in indirect tMNC co-cultures was significantly higher than in direct co-cultures at Day 2 and Day 3 ($p \leq 0.001$) as shown in figure 2A. Direct co-cultivation with tMNC resulted in a stable level of 6% ± 1% neuronal apoptosis and was therefore significantly lower than in post-hypoxic control cultures (Day 2: 46% ± 20%; Day 3: 85% ± 11%).

When tMNC were indirectly co-cultured neuroprotection was as pronounced as in direct co-cultures on the first day after hypoxia. Two and three days after application there was still a significant, but compared to control cultures reduced, protective effect in the indirect co-cultures while
protection in direct co-cultures was as distinct as on Day 1 (7% ± 8%).

The comparison of both application types using tMNC and CD133 showed that soluble factors seem to have strong therapeutic potential (Fig. 2A).

The positive influence of indirect co-cultivation on the amount of apoptotic cells, revealed by annexin-V detection, was also confirmed by typical patterns in the cleavage of PARP, a late marker of apoptosis (Fig. 3). In indirect co-cultures with tMNC and CD133 quantities of cleaved PARP were nearly at the same level on Day 1 post-hypoxia. On Day 2 and Day 3 neuroprotection by CD133 resulted in concentrations of cleaved PARP ranging in the level of the normoxic control (Fig. 3).

Only direct co-cultures of tMNC and CD133 displayed improved protection from necrosis/late apoptosis as revealed by Propidium Iodide labeling of post-hypoxic neuronal cells (Fig. 2C, D). Indirect co-cultivation did not reduce the percentage of necrotic/late apoptotic neuronal cells.

**tMNC localized in close proximity to post-hypoxic neuronal cells**

In direct co-cultures many tMNC were found in close spatial relation with post-hypoxic neuronal cells already at Day 1 (Fig. 4B). This became even more evident at later time points (Fig. 4D, F). At Day 3, the vast majority of tMNC was found adjacent to neuronal somata and processes (Fig. 4F). Interestingly, co-cultivation with tMNC seemed to have strong positive effects on the preservation of typical neuronal cell morphology as the formation of branched processes.

In contrast, in normoxic control cultures tMNC were evenly spread throughout the culture dish (Fig. 4A, C, E).
Figure 2

Effects of direct and indirect application of tMNC and CD133- on apoptosis and necrosis of post-hypoxic neuronal cells. Box plots represent the percentage of annexin-V positive post-hypoxic neuronal cells (A, B) and of PI positive post-hypoxic neuronal cells (C, D) in direct and indirect co-cultures. Co-cultures were performed with tMNC (A, C) and CD133- (B, D). The extents of apoptosis (annexin-V-binding) and necrosis (PI) were analyzed for three days after hypoxia.
In a second set of experiments we investigated different effects of direct and indirect co-culturing on cytokine secretion. Therefore, concentrations of CXCL9, CCL3, VEGF, G-CSF and GM-CSF were measured in normoxic and post-hypoxic mono-cultures, and co-cultures with tMNC and CD133⁺.

Except VEGF neuronal cells did not detectable amounts of either CXCL9, CCL3, G-CSF nor GM-CSF in mono-cultures. Both, tMNC and CD133⁺ cells expressed CCL3 and G-CSF but only CD133⁺ produced GM-CSF (Fig. 6).

In co-cultures with tMNC direct co-cultivation generated more pronounced effects than indirect co-cultivation, as seen for the relative up-regulation of CXCL9, GM-CSF (both, p ≤ 0.05) and CCL3 and the relative down-regulation of VEGF.

Only direct CD133⁺ co-cultivation had an impact on regulation of CCL3, VEGF and GM-CSF. Secretion of all three cytokines was markedly decreased compared to controls whereas indirect co-cultivation with CD133 had no effect. In contrast, the secretion of CXCL9 and G-CSF (p ≤ 0.01) was markedly induced by indirect co-cultivation with CD133⁺. For these cytokines direct co-cultivation showed no effect (G-CSF) or resulted in a remarkably reduced production (CXCL9) as compared to indirect co-cultivation with CD133⁺ (Fig. 6).

The experiment also revealed the influence of different HUCB-MNC-derived cell preparations on the cytokine secretion.

Most prominent, direct co-cultivation with tMNC significantly increased expression of CCL3 (p ≤ 0.05) whereas direct co-cultivation with CD133⁺ resulted in a relative reduction of this chemokine (p ≤ 0.01).

**Discussion**

It has been previously shown that HUCB derived MNC as well as nearly pure stem cell populations obtained from MNC are able to improve the clinical outcome of animals after MCAO [23]. In an *in vitro* model of neuronal hypoxia we discern cell populations within MNC being able to improve neuronal survival and disclose potential neuroprotective mechanisms.

First we studied the effects of tMNC application on the apoptotic status of post-hypoxic neurons. We found that direct application of tMNC results in preservation of neuronal morphology based on a constant protection from apoptosis (Fig. 2A).

For *in vivo* experiments it has been described that none or only a minority of systemically administrated cells were detected in the brain while large quantities were found in the spleen, in the lungs and in the blood of the animals.
Nevertheless, some studies showed that cell-treatment improved behavioral deficits [18,23,24]. This indicates the importance of soluble factors for neuroprotection. Our data obtained from indirect co-cultures strongly support this hypothesis. tMNC application was highly protective, although the anti-apoptotic effect was slightly weakened after two days (Fig. 2A). Probably, soluble factors act as “first aid” messengers while longer protection seems to demand close proximity between tMNC and neuronal cells. The comparison of direct application of tMNC to normoxic and post-hypoxic neuronal cells revealed that only post-hypoxic neuronal cells attracted tMNC (Fig. 4). The clustering of tMNC around neuronal cells could explain the enhanced protection from apoptosis in direct co-cultures with tMNC at Day 2 and Day 3 (Fig. 4, Fig. 2A). Spatial contiguity is possibly associated with higher concentrations of neuroprotective mediators in the micro-environment of injured neuronal cells. Post-

Figure 4
Distribution of tMNC in direct co-cultures. Phase contrast fluorescence micrographs of co-cultures of neuronal cells (unstained) and tMNC (green). Representative images show post-hypoxic neuronal cultures (B, D, F) in contrast to normoxic control cultures (A, C, E) over a time course of three days. Images illustrate the distribution of tMNC in relation to neuronal cells. White arrows point at spatial accumulation of tMNC which are clustered around neuronal somata and processes in post-hypoxic cultures (B, D, F). In normoxic cultures no clustering of tMNC was observed (A, C, E). The black arrow points at a conserved neuronal morphology including branched processes. Scale bars indicate 100 μm.
Cytokine profile in direct co-cultures with tMNC, CD133+ and CD133- on Day 3. **Control**: sum of cytokine concentration from single cultured (grey) post-hypoxic SH-SYSY cells and HUCB-MNC (light green = tMNC, light red = CD133+, not detectable = CD133-). **Co-culture**: direct co-culture of post-hypoxic SH-SYSY cells and HUCB-MNC (dark green = tMNC, dark red = CD133-, dark yellow = CD133+). Data are derived from four independent experiments and are expressed as pg/ml. * significant differences in cytokine concentrations of co-cultures compared to control cultures. Note different axis scaling.
**Figure 6**
**Effect of co-cultivation system (direct v. indirect) on cytokine secretion during co-cultivation of post-hypoxic neuronal cells with tMNC and CD133:**

**Control:** sum of cytokine concentration from single cultured (grey) post-hypoxic SH-SY5Y cells and HUCB-MNC (light green = tMNC, light red = CD133).

**Co-cultures:** co-culture of post-hypoxic SH-SY5Y cells and tMNC (direct: dark green, filled; indirect: dark green, striped), co-culture of post-hypoxic SH-SY5Y cells and CD133 (direct: dark red, filled; indirect: dark red, striped). Effects are displayed in percent as relative changes to control. **Mono-cultures:** absolute cytokine concentrations in supernatants of SH-SY5Y normoxic and post-hypoxic mono-cultures (white or grey bars, respectively) and tMNC (light green bars) or CD133 (light red bars). * significant changes to control. + significant differences between co-cultivation systems.
hypoxic neuronal cells were shown to significantly up-regulate the adhesion molecule ICAM-1 following hypoxia [25]. Hence, ICAM-1 (CD54) expression could underlay the observed co-localization via binding of LFA-1 (CD11) [26]. Besides adhesion molecules, chemotaxis could mediate this cellular co-localization. VEGF, known to exert chemotactic effects on monocytes [27], was produced by post-hypoxic SH-SY5Y cells (Fig. 6).

To focus the neuroprotective effects of stem cells we enriched or depleted CD133+ cells from HUCB-MNC. Purities of 97.38% for the stem cell preparation and a reduction of CD133+ up to 0.06% for the stem cell depleted fraction were achieved. In direct co-cultures CD133+ were applied to neuronal cells in quantities of $4.5 \times 10^3$ resembling 1% of the applied tMNC, since CD133+ account for about 1% of total cell number in tMNC preparations. Neuroprotective capacity of nearly pure CD133+ cells was comparable to tMNC (Fig. 1A). According to our analyses of cytokine production CD133+ cells did not secrete measurable cytokines neither in mono-cultures nor in co-cultures with injured neuronal cells (Fig. 5). Additional experiments using even tenfold elevated numbers of stem cells ($4.5 \times 10^4$) also revealed no detectable cytokine concentrations and did not result in an increased neuroprotective capacity (data not shown). Neuroprotection by the absence of measurable soluble mediators argues for a stem cell specific therapeutic mechanism through contiguity. Due to this assumption we did not include CD133+ in the investigation of indirect co-cultures in this study.

Surprisingly, CD133- cell fractions were also highly sufficient in protection from apoptosis. Therefore, the observed anti-apoptotic neuroprotective effects of tMNC in our experiments do not only relay on hematopoietic stem cell-specific mechanisms. This assumption is supported by the missing significance in the effect of CD133+ on necrotic/late apoptotic loss of post-hypoxic neuronal cells (Fig. 1B). tMNC and CD133+ significantly reduced the percentage of PI-positive neuronal cells, whereas CD133+ did not. Since CD133+ in indirect co-cultures were superior to tMNC in protection from apoptosis and because of very low amounts of CD133+ in this preparation, CD133- seem to mediate additional neuroprotective effects. Possibly, the separation process influenced the functionality of CD133+ cells. FACS analyses of the activation markers CD25, CD38, CD71 and HLA-DR on tMNC and on the separated CD133+ fraction did not reveal an increased population of activated cells in our preparations (data not shown). However, the expression of other activation molecules cannot be excluded, since this cell population displayed an enhanced secretion of cytokines like G-CSF, GM-CSF and CCL-3 (Fig. 6). We also cannot rule out that remaining CD34+ cells could account for the neuroprotective activity of the CD133+ fraction, since depletion did only reduce the number of CD34+ cells to 77% (Tab. 1).

The investigation of soluble mediators exhibited that hypoxia induced a significant increase of VEGF in neuronal cells (Fig. 6, [28]). VEGF is documented to inhibit pro-apoptotic signaling by Bad (BCL2 antagonist of cell death), and cleavage of caspase-3, and caspase-9 [29] and therefore can be claimed as an autocrine self-protection mechanism of damaged neuronal cells. The neuroprotective impact of tMNC and CD133- cells in direct applications was accompanied by prevention of VEGF production which is typically induced in post-hypoxic neuronal cells (Fig. 5).

Neuroprotective effects of cell application could be mediated by G-CSF that was found only in mono-cultures of CD133+ but not in mono-cultures of post-hypoxic SH-SY5Y cells. Schneider et al., 2005 [30] pointed out that human SH-SY5Y neuroblastoma cells express the G-CSF receptor and that activation by the neurotrophic G-CSF reduced NO-induced poly-ADP ribose polymerase

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<th>Table 1: Cellular subfractions of MNC</th>
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<td><strong>Cellular fractions of</strong></td>
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<td>I) Myeloid cells</td>
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<td>II) Lymphocytes (including stem cells)</td>
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<td>NK-cells</td>
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<td>CD133+/CD34-</td>
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Cellular fractions of HUCB-MNC after thawing of cryopreserved cells. Subpopulations of lymphocytes and stem/progenitor cells are expressed as percent of total lymphocytes.
(PARP) and caspase-3 cleavage. Our data support these observations. At Day 3 in indirect co-cultures supply of G-CSF by CD133+ was associated with cleaved PARP levels in the range of normoxic cultures (Fig. 3, Fig. 6). G-CSF levels in mono-cultures of tMNC were only slightly above detection limit and could not provide this anti-apoptotic effect (Fig. 6).

The decrease of cleaved PARP observed in post-hypoxic neuronal mono-cultures at Day 3 (Fig. 3) was probably induced by rising lack of energy due to increased rate of apoptosis (Fig. 1).

Noticeably, there are application-specific differences in the regulation of cytokine secretion in co-cultures with CD133+ fractions. Concentrations of CCL3 and G-CSF were significantly higher in indirect co-cultures than in direct co-cultures. Possibly these enhanced concentrations are responsible for a protection from apoptosis in indirect co-cultures similar to that in direct co-cultures at Day 3. Indirect co-cultivation with tMNC did not induce enhanced cytokine levels (Fig. 6) and at the same time did not exert the same neuroprotective effect on post-hypoxic SH-SY5Y neurons. This could be explained by spatial effects: paracrine released cytokines could be more effective than action of cytokines over a longer distance.

**Conclusion**

In this study we investigated human umbilical cord blood derived cell populations (tMNC, CD133+, and CD133-) according to their ability to protect post-hypoxic neuronal cells.

For different reasons, as the missing systemic effects and the disregard of brain cell interactions this in vitro system does only simplified reflect the action of MNC after hypoxic brain lesions in vivo. But taken this into account, our study delivers useful indications for the in vivo application of such cells:

So, since purified CD133+ fractions are not superior to total HUCB-MNC in mediating neuroprotective anti-apoptotic effects, expensive and time consuming stem cell separations are not necessarily needed to yield neuroprotective cell populations. Furthermore, our study underlines the importance of MNC derived soluble factors for the mediation of neuroprotective effects visible as prevention of neuronal cells from apoptosis. Therefore, future therapeutic approaches should focus on the sufficient supply of soluble anti-apoptotic mediators, to reduce post-hypoxic brain damage.

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**Methods**

**Preparation of HUCB samples and isolation of CD133+ cells**

HUCB samples of healthy full-term neonates were obtained in accordance with ethical prescripts immediately after delivery. Samples were processed and analyzed as described previously [25]. The total MNC (tMNC) fraction gained from Ficoll density gradient (Tab. 1) was stored in the gaseous phase of liquid nitrogen. Cellular sub-fractions of tMNC were characterized using CD3-Phycoerythrin (PE, Immunotech, Hamburg, Germany), CD14-Fluorescein isothiocyanate (FITC), CD16+56-PE (both, Becton-Dickinson, Franklin Lakes, NJ, USA), CD19-Allophycocyanin (APC) and CD45-FITC, (both, Beckman Coulter, Krefeld, Germany). Prior to use tMNC were thawed and stained with carboxy fluoresceindiacetate succinimidyl ester (CFSE, Invitrogen, Karlsruhe, Germany).

CD133 positive cells (CD133+) were isolated from HUCB-MNC using the MACS® immunomagnetic positive selection protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). The flow-through fraction was collected as negative fraction (CD133-) depleted of CD133+. Final populations were analyzed using a FACSCalibur flow cytometer equipped with the CellQuest™ software (both Becton-Dickinson, Franklin Lakes, NJ, USA) and characterized by the use of the following antibodies: CD133/2-PE, Miltenyi Biotech Bergisch Gladbach, Germany), CD45-FITC and CD34-APC (both Beckman Coulter, Krefeld, Germany). Isotype-identical monoclonal antibodies served as controls. CD133+ and CD133- fractions contained 97.38% and 0.06% CD133 positive cells, respectively.

Status of activation of tMNC and CD133+ was analyzed via FACS using the antibodies CD25-PE, HLA-DR (Major Histocompatibility Complex, class II, cell surface receptor)-APC (both Becton-Dickinson, Franklin Lakes, NJ, USA), CD38-PE and CD71-FITC (both Beckman Coulter, Krefeld, Germany).

**Differentiation and hypoxic induction of SH-SYSY cells**

For differentiation 0.9 × 10^6/cm² SH-SYSY cells were seeded in 16-mm-diameter wells (Greiner Bio-One, Frickenhausen, Germany). After 5 days Dulbecco’s Modified Eagle Medium (DMEM)/10 μM all-trans retinoic acid (Sigma-Aldrich, Steinheim, Germany)/15% fetal calf serum (PAN-Biotech, Aidenbach, Germany) media was changed into DMEM-Ham’s F12/10 μM RA/5 ng/ml Brain Derived Neuronal Factor [BDNF, Immunooltools Biolance GmbH, Hanover, Germany]/0.1% Human Serum Albumine [HSA, PAN Biotech GmbH, Aidenbach, Germany] for another 11 days. Media were exchanged every third day. Afterwards fully matured neuronal cells were cul-
tured under a hypoxic atmosphere (< 1% O2) for 48 hours [25]. Number of viable cells remained stable between Day 16 and Day 21.

Direct and indirect co-cultivation of post-hypoxic neuronal cells with tMNC, CD133+ and CD133− cell fractions

Subsequent to hypoxia, direct and indirect co-cultivation with tMNC, CD133+ or CD133− was carried out under normoxic conditions over a period of three days. Added tMNC, CD133+ (4.5 × 10^5 cells, both) and CD133− (4.5 × 10^3 cells) were dissolved in 500 μl co-culture medium (DMEM-Ham’s F12, 5 ng/ml BDNF and 0.1% HSA) and added to differentiated post-hypoxic neuronal SH-SY5Y cells cultivated in adequate volume of post-hypoxic medium. Cell ratio of post-hypoxic neuronal cells to cells cultivated under normoxic conditions was 1:15 and to CD133+ 1:0.15. For indirect co-cultivation tMNC or CD133− were added in cell impassable cell culture inserts with a pore size of 0.4 μm (Greiner Bio-One GmbH, Frickenhausen, Germany). Prior to co-cultivation with post-hypoxic neuronal cells, tMNC as well as CD133+ and CD133− were labeled with CFSE.

Cell viability assay of neuronal cells

Within direct and indirect co-cultures and control cultures the influence of tMNC, CD133+ and CD133− on neuronal viability was detected via i) Propidium Iodide (PI, Invitrogen, Karlsruhe, Germany) assay for necrosis and late apoptosis and ii) annexin-V assay (Becton-Dickinson, Heidelberg, Germany) for apoptosis. The PI- and annexin-V assays were performed in cell culture plates as described previously [25]. tMNC, CD133+ and CD133− were distinguished from annexin-V-PE or PI positive neuronal cells by the green CFSE staining.

Cytometric Bead Array for human apoptosis (CBA, Becton Dickinson, Erembodegem, Belgium) was used to quantify the apoptosis specific parameter cleaved Poly-ADP-Ribose-Polymerase I (PARP) in lysates of post-hypoxic neuronal cells after indirect co-cultivation with tMNC and CD133+. For cell lyses adherent neuronal cells were rinsed with PBS and incubated on ice in the supplied buffer for 20 minutes. For analyses pooled samples obtained from three independent experiments were used.

Cytokine profiling

For cytokine profiling supernatants of direct and indirect co-cultures were analyzed on Day 3. Supernatants of tMNC, CD133+ and CD133− mono-cultures and those of post-hypoxic neuronal cells were also investigated on Day 3. Cytokines were simultaneously measured using CBA for human soluble proteins (Becton Dickinson, Erembodegem, Belgium). Supernatants were screened for the following cytokines: CCL2, CCL3, CCL5, CXCL8, CXCL9 and for the growth factors basic Fibroblast Growth Factor (bFGF), Granulocyte Colony-Stimulating Factor (G-CSF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and Vascular Endothelial Growth Factor (VEGF). The detection limit was 20 pg/ml, except for CCL2 and VEGF (40 pg/ml).

Statistical analyses of data

Except for apoptosis and necrosis rates all results have been reported as mean ± SD. Statistical differences were analyzed by Student’s t-test or Mann-Whitney rank sum test. P values of ≤ 0.05 were considered statistically significant ( * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001). Apoptosis and necrosis rates were logit-transformed to obtain normally distributed quantities. The effects of time, experimental setting (post-hypoxia), experimental run and the investigated well were determined univariately, and, finally multivariately using a mixed-model approach with time and experimental setting as fixed effects and well and experimental run as random effects.

Cytokine concentrations of indirect and direct co-cultures with tMNC, CD133+ and CD133− were compared with the sum of the concentrations obtained after post-hypoxia of neuronal cells and corresponding tMNC, CD133+ and CD133− mono-cultures using a bootstrapping algorithm. Therefore, we added concentrations which were resampled from e.g. the experiment post-hypoxia and the tMNC mono-culture and compared the results with concentrations obtained from e.g. the experiment of indirect co-culture with tMNC. Results were compared with Student's t-test or Mann-Whitney rank sum test. P-values reported are based on 10,000 bootstrapping simulations.

Box plots (if applicable) and univariate analyses were determined using the software package SPSS (SPSS Inc., Chicago IL, USA). Mixed Model analyses were performed using PROC MIxed of the statistical software package SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Bootstrapping analysis was performed using the software package package "R" [31].

Authors’ contributions

DMR and SH coordinated and conducted all experimental work and wrote the manuscript. MK and TS helped to interpret the data and supported writing the manuscript. MS performed the statistical analysis. JB, WN and FE critically revised the manuscript.

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