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ASSAY OF SOME EMBRYONIC SIGNALING PATHWAYS IN A POSTAGGRESSIVE HYPOXIC SUFFERING MODEL ON NEUROBLASTOMA CELLS

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Introduction. Suffering (including injury) of the central nervous system leads to vascular lesions, followed by local hypoxic modification. And chronic ethanol abuse can favor nerve injuries. In this study we will evaluate the expression of genes involved in embryonic neural signaling pathways using a hypoxic suffering model in neuroblastoma cell cultures (in the case of chronic treatment with ethanol).

Material and methodsm. We performed a chronic ethanol exposure model in neuroblastoma cell cultures to which we added hypoxic stress induced by acute treatments with hypoxemic agents.

Results. We found differences in the gene expression of the molecules involved in embryonic-type signaling, both between neural cells chronically treated and untreated with ethanol, as well as between cells exposed and not exposed to hypoxemic conditions.

Discussions. The current study presents limitations of the tumoral nature of SK-N-SH cells and related to the difficulty of experimental reproduction of all phenomena that occur in neuraxial injury conditions.

Conclusions. Intercellular signaling pathways were investigated at the level of gene expression for SK-H-SH neural cells treated with ethanol and subjected to the hypoxia cell injury model. The following were observed: tendencies to reduce neuraxial scars (by inhibiting the expression of genes vimentin, nestin, cadherin, Olig2, GAFP molecules with a signaling role but, at least some of them, also involved in scar development), of neurite development and neurogenesis (by stimulating BMP4 gene expression), of neuroregeneration (by stimulating the expression of the Wnt gene), favoring (theoretical) the ability to differentiate into neurons or glial cells in the SK-N-SH line (by stimulating the expression of the CD133, NeuroD genes; by decreasing the inhibition of the Notch, Hey and Hes genes).

Keywords: hypoxia, chronic ethanol abuse, embryonic neural pathways, spinal cord injury, neural injury.

INTRODUCTION

Traumatology of the central nervous system (brain and spinal conrd injury) shows an upward trend in the modern world, where the problem of addictions (especially ethanol) continues to be current. The clinical experience in the Neuro-Muscular Recovery Department of Teaching Emergency Hospital "Bagdasar-Arseni" showed statistically significant differences in the favorable acute post-traumatic evolution of spinal-cord injury (SCI) patients chronic ethanol users, compared to SCI victims who had no history of chronic ethanol abuse[1]. For the understanding of these clinical findings we used (including) the molecular study of the signaling pathways of the immature (embryonic) type for neural cells.

In the present article we have presented an experimental molecular biology study (from the doctoral thesis component "Research on the consequences of chronic ethanol impregnation in the evolution of myelic lesions in patients with spinal cord injury") of hypoxic suffering in neural cell cultures with and without chronic ethanolic treatment.

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Regeneration of spinal nerve tissue (and from other neuraxial areas) could be possible with the participation of neural stem cells (NCS) located periventricular, hippocampal, retinal, in the meningeal system and in the area of the ependymal canal (of the spinal cord)[2].

During intrauterine development, most oligodendroglia are formed from pMN progenitor cells (expressing the transcription factor Olig2), located in the ventral region of the embryo[3,4]. PMN cells have the primordial tendency to transform into motor neurons and later differentiate into oligodendroglia[3,4]. In embryonic life, the precursor of the nervous system is the neural tube (developed from ectoderm), having in its ventral area the notochord (derived from mesoderm) that shapes the neural tube by means of sonic hedgehog signaling molecules (SHH-wnt)[4,5]. SHH also induces Olig2 expression in the anterior-posterior axis of the neural tube and maintains functional pMN cells throughout the entire period of neurogenesis[4].

CD133 (prominin-1) is a transmembrane (and cytosolic) protein found in neural stem cells, hematopoietic and tumor cells[6]. The transmembrane protein CDD133 can bind cholesterol molecules and be glycosylated, and its intracellular C-terminal domain can be phosphorylated (by the Src family of tyrosine kinases) which binds to (and activates) the p85 subunit of phosphoinositol kinase 3 (PI-3K) which it then activates Akt promoting the proliferation of glial stem cells[6]. CD133+ cells in the nervous system are immature, with a high potential for differentiation (into neuraxial cells or A neuroblasts) and proliferation[7–9]. CD133 protein accelerates growth and suppresses cell differentiation, being also involved in cell survival by influencing autophagy[6].

The Notch signaling pathway has different roles in neural cell differentiation processes (indirectly contributing to their cellular diversity) and in the establishment of glial typology, promoting neuronal apoptosis via the p53 protein pathway (in the developing brain) – being involved in the establishment of shape and size the nervous system[10]. The Notch receptor is proteolytically activated (through metalloprotease and presenilin action) with the release of its intracellular domain, which is then translocated to the nucleus, where it stimulates the transcription of the Hes (hairy/enhancer of split) and Hey (hairy ears, Y-linked) genes[10–12]. Decreased Hes expression appears to promote regeneration of progeny neurons in complete injury models after SCI in nematodes and vertebrates (Lampreda fish)[12]. The Hey family of transcription factors is also involved in the maintenance of neural stem cells during central nervous system development by forming Hes-Hey heterodimers (which suppress transcription of proneural genes)[11]. Decreased Notch expression leads to accelerated differentiation of spinal motor neurons, together with reduced Hes1, Hes5 and increased expression of Mash1, Neurogenin 1 and 2, thus the Notch pathway ensures the preservation of neural progenitor cell identity, along with involvement in the establishment of the specific neuronal subtype[10]. Notch signaling promotes the formation of glial cells: astrocytes (in the central and peripheral nervous system), retinal and telencephalic glial cells[10]. Inactivation of Hes 1, 3, 5 genes (transcribed by the Notch pathway) causes the differentiation of glocytes into neurons[10].

Regarding prenatal spatial development of the central nervous system, bone morphogenetic proteins 4 (BMP4) are part of a family of molecules that regulate the development of several cell types[13]. BMP4 regulates the stereotypical spatial distribution of neural tube-forming cells[13]. BMP4 and Smad1 (mothers against decapentaplegic homologue 1) are part of the BMP4/Smad1 pathway; and Smad1 activation, its nuclear accumulation and gene transcription occur after binding to the BMP receptor (BMPR), with histone 4 acetylation, causing axonogenesis by inducing transcriptional factors related to axonal regeneration; while BMP4 activates serine/threonine kinase 1, stimulates mitogen activated protein kinase (MAPK) and autocrine induction of growth factors[14].

The Wingless/Integrated (Wnt) protein family modulates important processes in various neuropathogenic conditions[15]. Wnt expression is increased in SCI, making the regeneration of cortico-spinal tracts difficult; some of the receptors of this signaling pathway being located at the level of axons, neurons, astrocytes, oligodendroglia, microglia in the injured area, which shows a possible involvement of Wnt in neurodegeneration, neuroinflammation and in the decrease of descending serotonergic innervation[15].

Nestin is a type VI intermediate filament protein in neural progenitor cells and neural stem cells, and is also a marker for these cells[16]. Neural precursor cells can express translational factors (such as neuronal differentiation 1-NeuroD1 and oligodendrocyte transcription factor 2- Olig2) that regulate their differentiation into neurons or glial cells[17]. Nestin interacts intracellularly with microfilaments and microtubules participating in the formation of

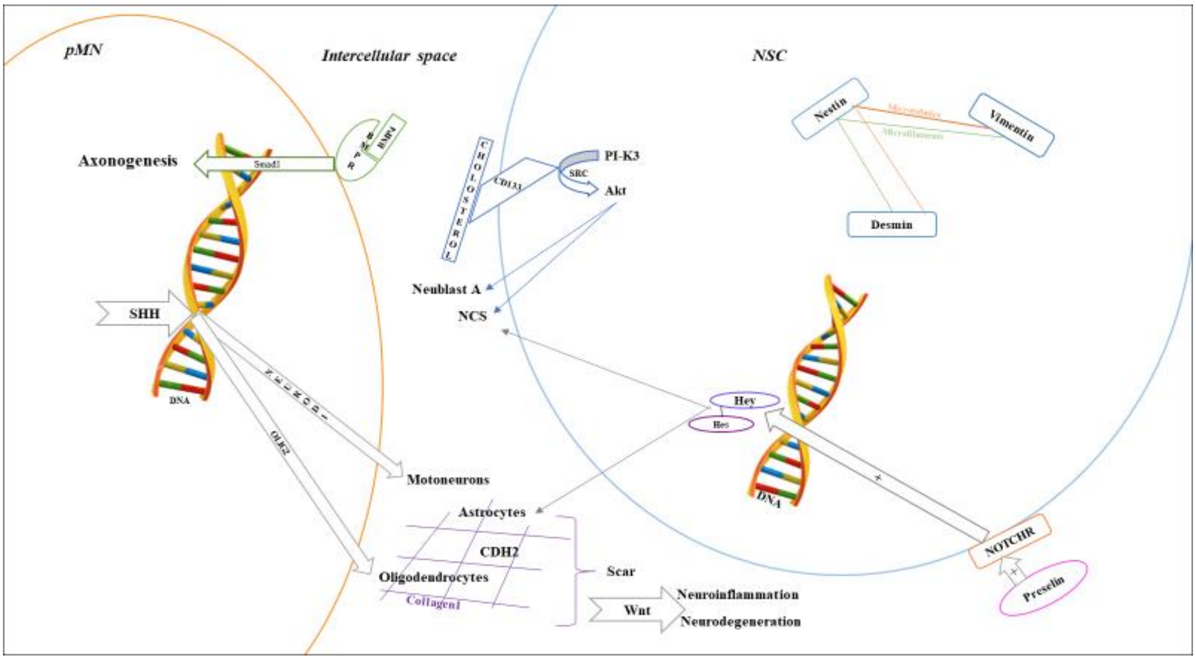

Figure 1. Embryonic signaling pathways involved in neuroregeneration.

heterodimers and small polymers through interaction with vimentin or desmin; and its gene expression is regulated by spatio-temporal factors that reduce intron amplification[16]. Adult neural cell injury increases nestin expression (possibly under the action of nerve growth factor or acidic fibroblast growth factor; by decreasing intercellular contact; by transcriptional modification at the level of introns; by remodeling of cellular intermediate filaments) which could indicate a regeneration, intensive or a regression to an immature phenotype[16,18]. However, neural progenitor cells in the ependymal (spinal cord) canal after SCI migrate to the injured area and differentiate into glial cells (astrocytes and oligodendroglia), not into neurons[19]. Reactive astrogliosis is implicated in neurodegeneration after SCI, as astrocyte precursor cells (positive for nestin and glial fibrillary acidic protein GFAP) have been identified starting within the first 30 hours after injury[20].

Glial scars formed in the area of spinal cord injury are thought to impede regeneration after SCI and are composed of reactive astrocytes and intermediate filaments: GFAP and vimentin[21–23]. However, glial scars formed after SCI can stabilize nerve tissue by protecting against neuroinflammation and promoting neuronal survival[21,23,24].

Cadherin 2 (CDH2) is a protein involved in scar formation in the subacute and chronic phases after SCI through the integrin–N-cadherin pathway, with reactive astrocytosis and collagen type 1 overexpression[25].

MATERIAL AND METHODS

The cell line used for the experiment is SK-N-SH and was maintained in Dulbecco's Modified Essential Medium (DMEM: F12) culture medium (Sigma, USA) supplemented with 10% inactivated fetal bovine serum (FBS) (Sigma, USA) at 37 ºC, 5% CO_2, in humid atmosphere. Cells were seeded in 25 cm^2 culture dishes at a density of 1×10^6.

After 24 hours, the cells were exposed to ethanol diluted in DMEM: F12 supplemented with 10% serum, in the following concentrations: 50, 100, 200 and 300 mM. The medium was changed daily for 10 days. During the study, cultures were observed under a microscope daily (for analysis of ethanol exposure-induced effects on cell morphology) and harvested after 10 days (for viability studies). Then, the study continued using the concentration of 50 mM ethanol diluted in the culture medium, in daily doses for more than 60 days (more than 9 weeks, to simulate chronic alcohol consumption).

The hypoxemic treatment was carried out following the treatments with Desferal/ Deferoxamine (DFX) (Novartis, Germany) and cobalt chloride $CoCl_2$ (Merck, Germany) in a concentration of 50 and 100 µM, for 24 hours, for cells not exposed to ethanol and for those with an exposure of more than 9 weeks. Isolation of total cellular RNA and verification of integrity was performed according to the following protocol. Unexposed and 50 mM ethanol-exposed SK-N-SH cells were harvested after more than 50 days of ethanol treatment. Cells were washed with TFS and centrifuged to obtain the cell pellet. Then the classic technique developed by Chomczynski and Sacchi was applied. Reverse transcription of RNA samples to cDNA has been applied to several genes: CDH2 (Hs00169953_m1), SHH (Hs01123832_m1), NOTCH1 (Hs01062014_m1), Hey1 (Hs01114113_m1), GFAP (Hs00157674_m1), BMP4 (Hs00370078_m1), Hes1 (Hs00172878_m1), NEUROD1 (Hs00159598_m1), WNT1 (Hs01011247_m1), OLIG2 (Hs00377820_m1), Nes (Hs007070120_m1), Vim (Hs00185584_m1).

RESULTS

The SHH signaling pathway was not expressed in any of the experimental variants with SK-N-SH cells (growth in simple culture medium/ chronic ethanol exposure medium/ under added hypoxic stress conditions); which could indicate that SK-N-SH neurons have a degree of differentiation that prevents them from regressing to a fully undifferentiated stage.

CD133 protein shows the ability to differentiate into neurons or glial cells, and its synthesis shows a higher value in SK-N-SH cells chronically exposed to ethanol compared to cells grown in plain culture medium. Experimental hypoxic conditions maintain the same trend of CD133 gene expression: in the case of treatment with deferoxamine 50/100 µM and cobalt chloride 50/100 µM.

Notch signaling is inhibited in the case of chronic ethanol treatment, being ameliorated in hypoxic conditions overloaded by deferoxamine 50/100 µM and cobalt chloride 50 µM treatment; and hypoxemic treatment with 100µM cobalt chloride appears to further inhibit Notch gene expression. We could say that chronic exposure to ethanol and hypoxic conditions have a tendency to stimulate the differentiation of neural cells to neurons in most hypoxemic situations. The expression of the Hey transcription factor gene from the Notch pathway is stimulated under conditions of chronic exposure to ethanol with the addition of hypoxic stress by treatment with deferoxamine 50/100µM and cobalt chloride 50/100µM; which could show the increased neural regenerative potential in the case of exposure to ethyl alcohol. The expression of the Hes transcription factor gene shows lower values (compared to SK-N-SH cells grown in the simple culture medium) in the case of chronic exposure to ethanol of SK-N-SH cells and in the situation of hypoxic stress addition by treatment with deferoxamine 50/ 100µM and CoCl250/100µM, possibly due to the tendency of immature glial cells to transform into neurons and the increased regenerative potential of motor neurons.

BMP_4 gene expression is superior in the case of chronic ethanol treatment; decreasing to undetectable values under hypoxic conditions produced by deferoxamine 50µM and $CoCl_2$ 50µM treatment and with stimulation in the case of increasing hypoxic stress by deferoxamine 100µM and $CoCl_2$ 100µM treatments, findings showing stimulation of growth factors and axonogenesis in the setting of chronic ethanol exposure, including to which hypoxia was superimposed

Wnt signaling pathway gene expression increases under chronic exposure to ethanol and additional treatment with deferoxamine 50/100 µM, while hypoxia induced by cobalt chloride 50/100 µM renders Wnt genes undetectable – which could to reflect neuroinflammation and neuro-degeneration following chronic alcohol consumption, but also the tendency to neuroregeneration that can occur in certain hypoxemic conditions.

The expression of cadherin2 genes is inhibited, both in the case of chronic exposure to ethanol and in hypoxemic conditions superadded by treatment with deferoxamine 50/100 µM and $CoCl_2$ 50/100 µM, a fact that may demonstrate the diminution of the ability to form glial scars in chronic treatment with ethanol and with hypoxic stress conditions added.

The NeuroD transcription factor gene has positive expression in both cell cultures chronically exposed to ethanol and the situation of adding hypoxic conditions by treatment with deferoxamine 50/100 µM and cobalt chloride 50/100 µM; a situation that could show the tendency of neural cells to become neurons in those conditions.

Olig2 transcription factor gene expression is stimulated in SK-N-SH cultures chronically exposed to ethanol and 100 µM cobalt chloride; with values becoming undetectable under hypoxic conditions produced by 50 µM cobalt chloride and 50/100 µM deferoxamine. The situation of the Olig2 gene could explain a tendency towards reactive gliosis in chronic alcoholism, which seems to disappear in the case of excess cellular hypoxia (also found in SCI).

Regarding cytosolic intermediate filaments, vimentin gene expression was undetectable in SK-N-SH cultures chronically treated with ethanol, with the addition of hypoxic stress (by deferoxamine 50/100 μM and $CoCl_2$ 50/100 μM treatments) which could to show decreased glial scar formation propensity for neurocytes abused by ethanol and additional hypoxia. Similarly, GFAP gene expression is inhibited in SK-N-SH cultures chronically exposed to ethanol and with hypoxic stress added by deferoxamine 50/100μM and CoCl2 50/100μM treatments; showing diminution of glial scars. Nestin gene expression was inhibited in SK-N-SH cultures chronically exposed to ethanol and added hypoxic conditions produced by 50/100 μM deferoxamine and 100 μM $CoCl_2$; while the treatment with $CoCl_2$ 50 μM produced the positivity of nestin gene expression. The nestin gene expression situation may show a tendency to decrease the production of glial scars in the case of chronic ethanol consumption, which is accentuated in the case of over-adding hypoxia.

DISCUSSIONS

Although research from the perspective of molecular biology is essential for understanding neural injury and recovery processes, the anatomical-physiological complexity of the human body makes it extremely difficult to create appropriate experimental models.

Thus, the current study presents limitations of the tumoral nature of SK-N-SH cells and related to the difficulty of experimental reproduction of all phenomena that occur in neuraxial injury conditions.

Table 1

Gene expression analysis of embryonic signaling pathway proteins

log 2^-ddCt	SHH	CD133	Notch1	Hey	BMP4	Hes	WNT	CDH2	Vim	Nestin	NeuroD	GFAP	Olig2
SK-N-SH	0	0	0	0	0	0	0	0	0	0	0	0	0
SK-N-SH _66>9w	0	1,602309	-0,56277	0,249199	0,330762	-0,63002	0,11891	-0,64334	-0,78537	-0,02283	1,59534	-0,59002	0,350692
SK-N-SH _>9w_DFX100	0	0,597933	-0,01992	0,12764	0,054145	-0,34607	0,571973	-0,91062	-0,75856	-0,29167	0,517872	-0,22388	0
SK-N-SH _>9w_DFX50	0	0,581367	-0,12744	0,231815	0	-0,33507	0,321737	-0,74764	-0,53936	-0,28498	0,643982	-0,22967	0
SK-N-SH _>9w_CoCl2_100	0	1,228194	-0,74628	0,272501	0,213441	-0,65508	0	-0,8637	-0,82925	-0,09815	1,154348	0	0,055981
SK-N-SH _>9w_CoCl2_50	0	1,756464	-0,30657	0,810926	0	-0,20796	0	-0,22354	-0,21382	0,267017	1,809928	-0,11158	0

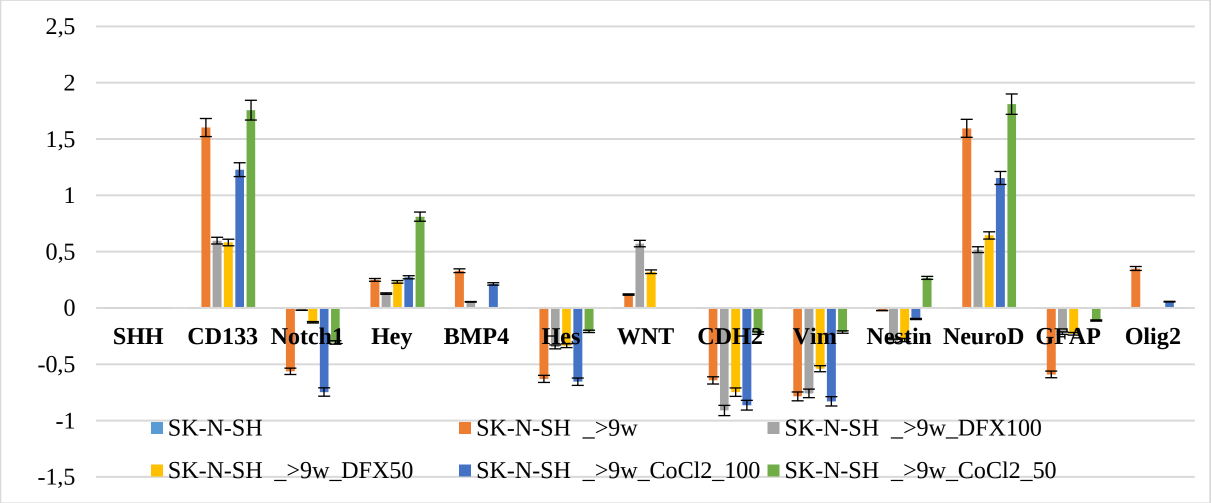

Figure 2. Gene expression analysis of proteins involved in embryonic signaling pathways.

CONCLUSIONS

Neuraxial injury presents many unknown pathophysiology, which makes neuro-motor recovery problematic. In these conditions, data from clinical observation are fundamental for molecular testing of the evolution of various injury models.

Intercellular signaling pathways were investigated at the level of gene expression for SK-H-SH neural cells treated with ethanol and subjected to the hypoxia cell injury model. The following were observed: tendencies to reduce neuraxial scars (by inhibiting the expression of genes vimentin, nestin, cadherin, Olig2, GAFP molecules with a signaling role but, at least some of them, also involved in scar development), of neurite development and neurogenesis (by stimulating BMP4 gene expression), of neuroregeneration (by stimulating the expression of the Wnt gene), favoring (theoretical) the ability to differentiate into neurons or glial cells in the SK-N-SH line (by stimulating the expression of the CD133, NeuroD genes; by decreasing the inhibition of the Notch, Hey and Hes genes).

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