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Modulation of cholinergic pathways and inflammatory mediators in blast-induced traumatic brain injury

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ABSTRACT

Cholinergic activity has been recognized as a major regulatory component of stress responses after traumatic brain injury (TBI). Centrally acting acetylcholinesterase (AChE) inhibitors are also being considered as potential therapeutic candidates against TBI mediated cognitive impairments. We have evaluated the expression of molecules involved in cholinergic and inflammatory pathways in various regions of brain after repeated blast exposures in mice. Isoflurane anesthetized C57BL/6 J mice were restrained and placed in a prone position transverse to the direction of the shockwaves and exposed to three 20.6 psi blast overpressures with 1–30 min intervals. Brains were collected at the 6 h time point after the last blast exposure and subjected to cDNA microarray and microRNA analysis. cDNA microarray analysis showed significant changes in the expression of cholinergic (muscarinic and nicotinic) and gammaaminobutyric acid and glutamate receptors in the midbrain region along with significant changes in multiple genes involved in inflammatory pathways in various regions of the brain. MicroRNA analysis of cerebellum revealed differential expression of miR-132 and 183, which are linked to cholinergic anti-inflammatory signaling, after blast exposure. Changes in the expression of myeloperoxidase in the cerebellum were confirmed by Western blotting. These results indicate that early pathologic progression of blast TBI involves dysregulation of cholinergic and inflammatory pathways related genes. Acute changes in molecules involved in the modulation of cholinergic and inflammatory pathways after blast TBI can cause long-term central and peripheral pathophysiological changes.

1. Introduction

Blast exposure has been described as one of the major factors involved in mild to moderate brain injury in service members returning from Iraq and Afghanistan which can lead to chronic neurological disabilities [1–4]. Acute changes in the central and peripheral nervous systems after blast TBI can exacerbate the pathological outcomes resulting in long-term chronic effects [5,6]. Neuroinflammation including cross-talk between central and peripheral immune systems is considered to be a primary event after blast exposure exacerbating the brain injury [6,7]. Inflammation and innate immune responses are primarily regulated by neural mechanisms [8,9]. In particular, cholinergic systems involving the neurotransmitter acetylcholine and the enzyme acetylcholinesterase (AChE) have been proposed as components of an anti-inflammatory pathway regulating neuroimmunomodulation [8–11].

Recently we reported regional specific alterations in the brain AChE activity after repeated blast exposures [12]. AChE inhibitors are possible therapeutic candidates against Alzheimer's disease and TBI [13–15]. In this study, we analyzed the expression of cholinergic and inflammatory related genes in different regions of the brain of repeated blast exposed mice using cDNA microarray. We also analyzed the microRNA expression profile in the cerebellum of blast exposed mice. MicroRNAs are endogenous tissue specific non-coding ribonucleic acids of approximately 18–26 nucleotides which modulate gene expression by binding to complementary mRNA, either targeting degradation or inhibiting translation, potentially play major roles in neuropathophysiology.

2. Materials and methods

2.1. Materials and animals

Trizol reagent, Tris–Bis gradient gels (4–12%), protein molecular weight markers, and SDS–PAGE running and transfer buffers were

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purchased from Invitrogen Life Technology (Carlsbad, CA); tissue protein extraction reagent and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL); acetylthiocholine, tetra monoisopropyl pyrophosphortetramide (iso-OMPA), and 4,4′-dipyridyl disulfide, 4,4′-dithiodipyridine (DTP) were purchased from Sigma–Aldrich (St. Louis, MO); polyvinylidene difluoride (PVDF) membrane and anti-myeloperoxidase (MPO) antibody were purchased from Millipore (Billerica, MA). C57BL/6 J mice (male, 8–10 weeks old, 21–26 g) were purchased from Jackson Laboratory, Bar Harbor, ME. Animal experiments were performed at Walter Reed Army Institute of Research (WRAIR) in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council Publication, 1996 edition) with an approved Institutional Animal Care and Use Committee protocol. Isoflurane anesthetized mice were exposed to 20.6 psi blast overpressure for three times with 1–30 min intervals as described earlier [5,12,16].

2.2. Brain acetylcholinesterase activity assay

Brain samples collected at various time points (3, 6, 24 h and 3, 7, 14 days) were dissected into different parts (frontal cortex, hind cortex, hippocampus, cerebellum, mid brain and medulla) and homogenized with tissue protein extraction reagent at 4 °C using a tissue homogenizer and centrifuged. AChE activity in the supernatant of brain extracts were measured by using modified Ellman assay with 1 mM of acetylthiocholine substrate and 0.2 mM DTP as chromogen in the presence of 4 μM of iso-OMPA, a butyrylcholinesterase inhibitor, as described earlier [12,17–19]. Brain AChE activity was expressed as milliunits/mg protein.

2.3. Microarray analysis

Various regions of the brain (frontal cortex, cerebellum, mid brain, and hippocampus) of sham and repeated blast exposed mice at 6 h time point after the last blast exposure were collected and total RNA was isolated using Trizol reagent according to manufacturer's protocol. The concentration and purity of RNA were determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (5 μg) was labeled using the Agilent Low Input Quick Amp labeling Kit in conjunction with the Agilent two-Color Spike-Mix according to the RNA Spike-In Kit protocol and amplified in a thermal cycler (Mycycler, Bio-Rad Laboratories, Hercules, CA). Labeled RNA samples were subjected to fragmentation followed by 17 h hybridization against universal mouse reference RNA (Stratagene, La Jolla, CA) using the Agilent Gene Expression Hybridization Kit. Agilent 60-mer whole mouse genome 44 K oligo microarrays (Agilent Technologies, Santa Clara, CA) printed with Agilent SurePrint technology were used for microarray analysis as outlined in the Two-Color Microarray-Based Gene Expression Analysis (version 6.5) protocol. Microarray slides were scanned using an Agilent G2565CA fluorescence dual laser scanner for Cy3 and Cy5 excitation and the generated data were feature extracted using default parameters in Agilent Feature Extraction Software (version 10.7.1). GeneSpring 10.1 Software was used to carry out the data filter and normalization.

2.4. Western blot analysis of MPO expression

Total protein was extracted from the cerebellum of sham and repeated blast exposed mice at 6 h time point using tissue protein extraction reagent and the total protein content was estimated by using BCA assay kit. Equal aliquots (30 μg) of protein extracts were separated on 4–12% SDS–PAGE, transferred to PVDF membranes and probed with anti-MPO antibody. Blots were developed by using chemiluminescent substrate, photographed by AlphaImager (CellBioSciences, Santa Clara, CA) and quantified by using ImageJ software.

2.5. MicroRNA analysis

For the microRNA analysis, total RNA was extracted from cerebellar region of sham and repeated blast exposed mice (6 h time point after the last blast exposure) as described above followed by analysis by μParaflo™ MicroRNA microarray at LC Sciences (Houston, TX). Briefly, 4–8 μg total RNA was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) and the small RNAs (<300 nt) isolated were 3′-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly (A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a μParaflo microfluidic chip using a micro-circulation pump. On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA (from miRBase, version 16) or other RNA (control or customer defined sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using photogenerated reagent chemistry. After RNA hybridization, tag conjugating Cy3 and Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B, Molecular Device, Sunnyvale, CA) and digitized using Array-Pro image analysis software. Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression).

2.6. Data and statistical analysis

Statistical analysis of brain AChE enzyme activity was performed by using GraphPad Prism software with Mann–Whitney test. The statistical analysis of the microarray data was performed by using GeneSpring 10.1 Software. Differentially regulated genes (between sham control and blast exposed samples) were selected using Welsh's *t*-test analysis ($p < 0.05$), followed by the Benjamini–Hochberg multiple correction test to find genes that varied between control and blast exposed samples with a false discovery rate of 5%. To account for the small sample size, we used the reference design and filtered for genes with signal intensities that are twice the standard deviation of the background intensity levels. We determined that by performing gene-by-gene *t*-tests, for a samples size of 3% and 5% false discovery rate and a standard deviation of 0.5, the power is 75%. We also applied pathway and gene ontology analyses that offer extra power because it is statistically unlikely that a larger fraction of false positive genes end up in one specific pathway.

3. Results

3.1. Summary of AChE activity changes in different regions of brain after blast exposure

Changes in AChE activity in different regions of brains following repeated blast exposure have been reported earlier and the summary is shown in Fig. 1 [12]. Except for frontal cortex, all other brain regions of blast exposed mice showed an acute decrease in the activity of AChE. In the cerebellum and midbrain regions, a significant increase in AChE activity was observed at 3 days, while the

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