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Contribution of systemic factors in the pathophysiology of repeated blast-induced neurotrauma

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HIGHLIGHTS

- Repeated blast exposure increases the activation of platelets.
- Activation of leukocytes was observed after blast exposure.
- Vasoconstriction was evident in the cerebral cortex after blast exposure.
- Platelet/leukocyte targeted drugs to mitigate brain injury after blast exposure.

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ABSTRACT

Blast-induced traumatic brain injury is complex and involves multiple factors including systemic pathophysiological factors in addition to direct brain injuries. We hypothesize that systemic activation of platelets/leukocytes plays a major role in the development and exacerbation of brain injury after blast exposure. A mouse model of repeated blast exposure that results in significant neuropathology, neurobehavioral changes and regional specific alterations in various biomolecules in the brain was used for the proposed study. Activation of platelets was evaluated by flow cytometry and serotonin content was analyzed by ELISA. Expression of myeloperoxidase was analyzed by Western blotting. Histopathology of the brain was used to assess blast-induced cerebral vasoconstriction. The data showed an increase in the activation of platelets at 4 h after repeated blast exposures, indicating changes in platelet phenotype in blast neurotrauma. Platelet serotonin concentration showed a significant decrease at 4 h after blast with a concurrent increase in the plasma serotonin levels, confirming the early onset of platelet activation after repeated blast exposures. Blood, plasma and brain myeloperoxidase enzyme activity and expression was increased in repeated blast exposed mice at multiple time points. Histopathological analysis of the brains of blast exposed mice showed constriction of blood vessels compared to the respective controls, a phenomenon similar to the reported cerebral vasoconstriction in blast affected victims. These results suggest that repeated blast exposure leads to acute activation of platelets/leukocytes which can augment the pathological effects of brain injury. Platelet/leukocyte targeted therapies can be evaluated as potential acute treatment strategies to mitigate blast-induced neurotrauma.

1. Introduction

Blast-induced traumatic brain injuries (blast TBI) sharply increased in recent military conflicts due to asymmetric warfare and high use of improvised explosive devices and hand held grenades by insurgents. Definition of how the blast shockwaves enter the brain and cause biochemical and molecular changes leading to TBI or trigger secondary pathological processes and long-term neurobehavioral deficits is still elusive. Proposed major mechanisms of blast-induced TBI involve direct transmission of the shockwaves through the skull, or through orifices of eyes, nose and ear, and transmission of transient pressure waves from torso to brain [5,8,18]. Clinical and animal studies show that blast exposure leads to blood–brain barrier (BBB) breakdown along with neuronal/axonal/glial damage which are thought to be linked to behavioral deficits [4,25,26].

Recently, it has been reported that the severity of blast neurotrauma was higher following blast exposure to the torsoes of subjects with head protection than was seen following head-only

Abbreviations: Blast TBI, blast-induced traumatic brain injury; PMNs, polymorphonuclear neutrophils; BBB, blood–brain barrier; MPO, myeloperoxidase; PRP, platelet rich plasma; PGE_1, prostaglandin E_1; BCA, bicinchoninic acid; ELISA, enzyme linked immunosorbent assay; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; H & E, hematoxylin and eosin; LPA, lysophosphatidic acid.

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exposures [4,16]. Furthermore, blast neurotrauma was significantly attenuated by torso protection but not by head protection, indicating that blast mediated systemic responses play a critical role in the development of blast neurotrauma [4,16]. Previous studies from our laboratory in rats using Kevlar vest, which protected the thorax and abdomen, also showed significantly reduced mortality rates and brain injury after blast exposure [20]. The nature of the systemic factors that induce blast TBI is still obscure. Hemostatic abnormalities, cerebral vasospasm, pseudoaneurysm and neuroinflammation have been identified as key pathophysiological features of blast neurotrauma [1,19]. It has been suggested that altered blood components are involved in the development of blast TBI/cerebral vasospasm [1]. For example, activated blood components can promote BBB breakdown, neuroinflammation, and cerebral vasospasm which can augment the brain injury process leading to blast TBI. The contribution of systemic response along with the proposed torso to brain hydraulic overpressure transmission in the development of blast neurotrauma and cerebral vasospasm warrants further investigation [4,23].

We established a tightly-coupled single and repeated blast injury model in mice which resulted in significant neuropathology, neurobehavioral changes and brain regional specific alterations in various biomolecules [27,29]. Utilizing this model, we have evaluated the changes in systemic responses focusing on platelets and polymorphonuclear neutrophils (PMNs) activation process, which is presumed to be involved in the development and exacerbation of brain injury to outline their role in blast neurotrauma.

2. Materials and methods

2.1. Materials

Tissue protein extraction reagent and bicinchoninic acid (BCA) protein assay kits were purchased from Pierce Chemical Co. (Rockford, IL); serotonin research enzyme linked immunosorbent assay (ELISA) kit was purchased from Rocky Mountain Diagnostics, Inc. (Colorado Springs, CO); myeloperoxidase (MPO) enzyme assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI). Anti-mouse MPO antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Prostaglandin E_1 (PGE_1) and monoclonal antibody against β-actin were purchased from Sigma–Aldrich (St Louis, MO). PE-conjugated anti-mouse $\alpha_{IIb}\beta_3$ antibody was purchased from Emfret Analytics (Germany).

2.2. Repeated blast injury mouse model

Animal procedures were performed at the 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. Briefly, groups (n = 4–6) of anesthetized mice (male C57BL/6J mice, Jackson Laboratory, Bar Harbor, ME; 8–10 weeks old, 22–26 g weight) were exposed to 20.6 psi blast overpressure three times with 1–30 min intervals as reported earlier [29]. At 4, 24, and 72 h after the last blast exposure, blood and brain were collected from sham and blast exposed animals.

2.3. Flow cytometric analysis of platelet activation

Blood from the sham and blast exposed mice was collected in acidified citrate dextrose solution (1:10) containing 1 μg/ml of PGE_1. Platelet rich plasma (PRP) was separated by centrifugation at 800 rpm for 10 min at 22 °C. Platelets were pelleted from PRP by centrifugation at 2000 rpm for 10 min at 22 °C, washed twice with Tyrode buffer (137 mM NaCl, 12 mM $NaHCO_3$, 2.5 mM KCl pH7.2) and resuspended in Tyrode buffer containing 0.35% bovine serum albumin, 0.1% glucose, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. The washed platelets (2×10^5/ml) from sham and blast exposed animals were incubated with 0.1 U/ml of thrombin for 15 min at room temperature followed by incubation with PE-conjugated anti-$\alpha_{IIb}\beta_3$ antibody (1:100) for 15 min. Data from 5000 PE-positive platelets were acquired using a Guava Technologies flow cytometer (Millipore, Billerica, MA) and analyzed by the FlowJo software (Tree Star, Ashland, OR).

2.4. Estimation of serotonin

Serotonin content in the plasma, platelets and frontal cortex extracts was analyzed by highly specific competitive inhibition ELISA kits according to the manufacturer's protocols. Briefly, blood was collected from sham and blast exposed animals in acidified citrate dextrose solution (1:10) containing 1 μg/ml PGE_1 and 0.1% ascorbic acid. The platelets and plasma were separated from other blood components as described earlier, and the platelets were lysed in deionized water containing 0.1% ascorbic acid. The frontal cortex samples from sham and blast exposed animals were homogenized with tissue protein extraction reagent containing 0.1% ascorbic acid. Diluted serotonin standards, plasma, platelet lysates and frontal cortex extracts were acylated using acylation reagents provided with the ELISA kit at room temperature for 30 min with shaking. Aliquots of acylated standards and samples were incubated with serotonin antiserum overnight at 4 °C in a serotonin antigen pre-coated microtiter plate. The unbound antibody was washed with washing buffer, followed by incubation of the microtiter plate with anti-rabbit IgG conjugated with peroxidase for 30 min at room temperature. The bound enzyme was quantified using tetramethylbenzidine substrate and the absorbance was measured at 450 nm using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentrations of serotonin in the plasma, platelets, and frontal cortex extracts were calculated from the serotonin standard curve. This ELISA kit is 100% specific for serotonin with minor cross-reactivity to few other amino acid derivatives like tryptamine (0.2%), melatonin (0.03%), 5-hydroxyindole acetic acid, phenylalanine, and histidine etc. (all <0.002%).

2.5. Immunoblotting of myeloperoxidase (MPO)

Blood samples and frontal cortices of sham and blast exposed animals were lysed/homogenized with tissue protein extraction reagent containing protease inhibitor cocktail. Equal amount of proteins (~30 μg) from blood and frontal cortex samples of sham and blast exposed mice were separated on 4–20% gradient SDS-PAGE gels, transferred to polyvinylidene membranes and blocked with 4% non-fat dry milk for 1 h at room temperature. The blots were incubated with anti-mouse MPO monoclonal antibody (1:1000) overnight at 4 °C. The blots were washed with phosphate buffered saline containing 0.1% Tween-20, and incubated with anti-mouse secondary antibody conjugated to peroxidase (1:5000) for 1 h at room temperature. The blots were washed and the chemiluminescence was developed by using ECL detection reagent and photographed with Alpha Imager (Cell BioSciences, Santa Clara, CA). The resultant blots were stripped with stripping buffer and re-probed with anti-β actin antibody as the loading control. The images were quantified by using ImageJ software.