Inflammation and the Emerging Role of the Toll-Like Receptor System in Acute Brain Ischemia

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Background and Purpose—Systemic administration of cytosine-guanine (CpG) oligodeoxynucleotides provides neuroprotection against subsequent cerebral ischemic injury. We examined the genomic response of leukocytes and brain cells after ischemia in the context of CpG preconditioning.

Methods—RNA was isolated from circulating leukocytes and ischemic cortex 3 and 24 hours after middle cerebral artery occlusion after CpG or saline pretreatment and subjected to microarray analysis. Genes uniquely upregulated in CpG-pretreated mice were examined for overrepresented transcriptional regulatory elements.

Results—CpG preconditioning induced a novel response to middle cerebral artery occlusion within circulating leukocytes that was dominated by natural killer cell-associated genes and the GATA-3 transcriptional regulatory element. Preconditioning also caused a novel brain response to stroke that was dominated by Type I interferon, interferon-associated genes, and transcriptional regulatory elements.

Conclusion—CpG preconditioning invokes novel leukocyte and brain responses to stroke. In this, CpG may be a unique preconditioning agent, coordinating peripheral and brain responses to protect against ischemic injury. (Stroke. 2009; 40[suppl 1]:S34-S37.)

Key Words: inflammation ■ hypoxia-ischemia ■ stroke

Bacterial nonmethylated cytosine-guanine (CpG) oligodeoxynucleotide motifs alert the body to infection through activation of Toll-like receptor 9 (TLR9). In mice, TLR9 is expressed by B cells, plasmacytoid dendritic cells, macrophages, microglia, and astrocytes. TLR9-activated cells produce the proinflammatory cytokines tumor necrosis factor α, interferon α, and interleukin (IL)-12. These cytokines further activate monocytes, neutrophils, natural killer cells (NK cells), and T cells, facilitating a coordinated inflammatory response to pathogen invasion.

Pre-exposure to CpG reprograms the cellular response to subsequent TLR stimulation. Unlike naïve cells, macrophages pretreated with CpG do not generate tumor necrosis factor α in response to TLR4 stimulation, instead generating IFNβ.1 Furthermore, systemic administration of CpG increases resistance to polymicrobial sepsis.2 Hence, pre-exposure to CpG redirects both cellular and systemic responses to subsequent TLR stimulation.

Systemic administration of CpG also protects the brain from subsequent ischemic damage.3 Such “CpG preconditioning” is time- and dose-dependent and requires tumor necrosis factor α. The precise mechanisms responsible for CpG preconditioning are not well understood but likely involve both direct cellular processes and coordinated systemic responses that minimize ischemic damage.

We hypothesize that CpG preconditioning reprograms the response of the brain and the peripheral immune system to subsequent stroke. We provide evidence for such reprogramming and consider its potential neuroprotective consequences.

Materials and Methods

Mice
C57Bl/6 mice (male, 8 to 10 weeks) were obtained from Jackson Laboratories (West Sacramento, Calif). All mice were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal protocols met National Institutes of Health guidelines with the approval of the Oregon Health and Science University Institutional Animal Care and Use Committee.

Drug Treatments
CpG oligodeoxynucleotide 1826 (20 to 40 µg; 200 µL; Invivogen; San Diego, Calif) or saline was administered by intraperitoneal injection 72 hours before MCAO.

Surgery
Mice were anesthetized with isoflurane and ischemia was induced by MCAO as published previously.17 Cerebral blood flow was monitored with laser Doppler flowmetry and temperature was maintained at 37°C. After surgery, mice were kept for 24 hours on a heating pad with access to soft food and water.

RNA Isolation
Mice were anesthetized and blood was obtained through retro-orbital puncture. Animals were perfused with saline and, under RNase-free
conditions, a 1-mm section was removed for infarct area analysis. The ipsilateral cortex region from the frontal 4 mm was snap-frozen. Total RNA was isolated from the blood using the Qiagen PAXgene Blood RNA Kit and from the brain using the Qiagen RNeasy Lipid Mini Kit (Qiagen Inc). RNA from individual animals was hybridized to single arrays.

**GeneChip Expression Analyses**

Microarrays were performed in the Affymetrix Microarray Core of the Oregon Health & Sciences University Gene Microarray Shared Resource. RNA samples were labeled using the NuGEN Ovation Biotin RNA Amplification and Labeling System_V1. Quality-tested samples were hybridized to the MOE430 2.0 array and processed with Affymetrix GeneChip Operating Software. Data were normalized using the Robust Multichip Average method. Normalized data were analyzed by multivariate analysis of variance for each gene. Probability values were adjusted for multiple comparisons using the Hochberg and Benjamini method. Significance was determined by *P* < 0.05 and fold change ≥2 for blood analyses and ≥1.5 for brain analyses.

**Transcriptional Regulatory Network Analysis**

For our reference comparison group, we identified putative TREs in the upstream sequence of transcripts represented on the MOE430 Affymetrix gene chip using TRANSFAC PRO database version 10.4. We then determined the overrepresented TREs in the uniquely upregulated gene cluster compared with the reference group using Promoter Analysis and Interaction Network Toolset version 3.5.

**Results**

**Cytosine-Guanine Preconditioning Induces a Natural Killer Cell-Associated Peripheral Response to Stroke**

We evaluated RNA from blood leukocytes 24 hours after middle cerebral artery occlusion (MCAO) using Affymetrix oligonucleotide microarrays. We found 422 genes to be differentially regulated in CpG-pretreated animals relative to saline. We next identified overrepresented transcriptional regulatory elements (TREs) in the genes uniquely increased in CpG-preconditioned animals. In those genes for which the upstream sequence was available for analysis (234), a single TRE, GATA-3, was overrepresented with an adjusted probability value = 0.118. A network depiction of interactions between GATA-3 and genes in the CpG-preconditioned cluster is displayed in Figure 1. GATA-3 is linked to 53% of the genes within this upregulated cluster (124 of 234). GATA-3 plays a critical role in the development of natural killer (NK) cells. A literature review identified 24 of the upregulated genes as NK cell-associated: Klr5, Klr7, Klr8, KlrA10, KlrA18, KlrA22, KlrB1a, KlrB1c, KlrB1f, KlrC1, KlrC2, KlrE1, KlrG1, KlrK1, Rantes, Cma1, Eomes, Fasl, Gzmb, Il2rb, Ncr1, Ndg1, Prf1, and T-bet. CpG activates NK cells indirectly through IL-12 released from activated dendritic cells. Serum IL-12 levels were significantly increased 24 hours after MCAO in preconditioned animals (data not shown). Together, our data demonstrate that CpG preconditioning induces a novel, systemic NK cell response to stroke.

**Cytosine-Guanine Preconditioning Induces a Type I Interferon-Associated Brain Response to Stroke**

We evaluated RNA from ischemic cortex 24 hours after MCAO using Affymetrix oligonucleotide microarrays. We found 223 genes to be differentially regulated in CpG-pretreated animals relative to saline. We next identified overrepresented TREs in the genes uniquely upregulated in CpG-preconditioned animals. In those genes for which upstream sequence was available for analysis (136), we identified 4 overrepresented TREs with an adjusted probability value < 0.1. Notably, each TRE was Type I IFN-associated (IRF, IRF8, ISRE, 3-hydroxy-3-methylglutaryl-1-Y). A network depiction of interactions between the identified TREs and the genes in the CpG-preconditioned cluster is displayed in Figure 2. The IFN-associated TREs are linked to 64% of the genes within this upregulated cluster (88 of 136). A literature review identified 12 of the upregulated genes as Type I IFN-associated: Osas1a, MHC class I (H2-D1, H2-K1, H2-L, H2-Q6), Ifi203, Ifi204, Ifi205, Ifi207, Isg2011, Lmp7, and Psmb9). Thus, an altered signaling cascade involving Type I IFNs exists in the brain after stroke in CpG-preconditioned mice.

**Discussion**

We report the first evidence that CpG preconditioning alters the genomic response to stroke in circulating leukocytes and in the brain. We demonstrate a distinct pattern of NK cell activity in the blood and a clear enhancement of Type I IFN signaling in the brain after MCAO. This pattern of upregulated gene expression underscores a unique response to brain ischemia that may actively protect the brain from injury.

Cpg preconditioning induced a novel genomic response in blood leukocytes that was evident 24 hours after stroke. Of those genes uniquely upregulated in preconditioned animals, a majority contained the GATA-3 TRE, which is
CpG shifts the cellular response to subsequent stimulation of endogenous ligands such as HSP60, released after stroke, and potentially leads to suppressed cytotoxic tumor necrosis factor α and enhanced neuroprotective IFNβ.

Alternatively, the systemic increase in NK cell activity may explain the Type I IFN shift in the brain. NK cells promote the release of IFNα from plasmacytoid dendritic cells in a CpG- or IL-12-dependent manner. Hence, pretreatment with CpG may activate dendritic cells to produce IL-12, thereby activating NK cells which, in turn, induce plasmacytoid dendritic cells to produce IFNα.

We have shown that CpG preconditioning reprograms the peripheral and central responses to stroke. The appearance of a novel NK cell and IFN genomic “fingerprints” after ischemia indicates that CpG preconditioning fundamentally changes the body’s inflammatory response to stroke. This is consistent with our previous reports of reprogramming in which ischemic and lipopolysaccharide preconditioning induce novel, protective sets of gene transcripts after stroke. 

CpG appears to be a unique preconditioning agent, coordinating both systemic and central immune components to actively protect the body from ischemic injury.

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**Disclosures**

OHSU, Dr Stenzel-Poore, and Ms Stevens have a financial interest in Neuroprotect, Inc. This potential conflict of interest has been reviewed and managed by OHSU and the Integrity Program Oversight Council. There are no other conflicts to report.

**References**


**Figure 2.** Type I INF-associated TREs are overrepresented in the brain 24 hours after stroke in CpG-preconditioned mice. A Promoter Analysis and Interaction Network Toolset-generated hypothesis gene–TRE network shows the relationships between the genes uniquely upregulated by CpG preconditioning 24 hours after MCAO and the TREs shared in common. Genes are depicted as ovals. Probability value threshold set at 0.1.
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