Journal of Neuroimmunology 238 (2011) 19–33

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Cytokines regulate neuronal gene expression: Differential effects of Th1, Th2 and monocyte/macrophage cytokines

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ARTICLE INFO

Article history:
Received 27 April 2011
Received in revised form 16 June 2011
Accepted 17 June 2011

Keywords:
Cytokines
Glia
Inflammation
Multiple sclerosis
Neurodegeneration

ABSTRACT

Inflammatory mediators, including cytokines, contribute to neuronal and axonal dysfunction and cell death. To examine the roles of cytokines in pathogenesis and regeneration in the central nervous system (CNS), we analyzed effects of cytokines on early gene regulation (6 h) in neuronal cultures, employing gene arrays. Our hypothesis is that neuronal gene expression is differentially regulated *in vitro* by cytokine mixtures typical of Th1 and Th2 T cells and monocytes/macrophages (M/M). Th1 and M/M cytokines showed similar patterns for regulation of numerous pathways including cytokine-receptor interactions, MAP kinase, toll like receptors, apoptosis, PPAR signaling, cell adhesion molecules (CAMS), antigen processing, adipocytokine, and JAK-STAT signaling. M/M cytokines uniquely regulated genes in T cell, B cell and ECM receptor signaling pathways. Th2 cytokines had few effects on pathways regulated by Th1 and MM cytokines, but uniquely regulated genes related to neuroactive ligand-receptors and calcium. Th1 and MM cytokines markedly upregulated a wide array of cytokine-related genes. Notably, M/M cytokines uniquely upregulated G-CSF, GM-CSF, CXCL5 and lymphotactin (Xcl1). Th2 cytokines did not upregulate cytokine-related genes, with the exception of CCL11 and FMS-like tyrosine kinase 1, a VEGF receptor. In neuroactive ligand-receptor pathways, Th1 and M/M cytokines upregulated gene expression for tryptophan hydroxylase. Th1 cytokines upregulated gene expression for GABA A receptor, delta, while Th2 cytokines downregulated GABA A receptor, gamma 3. Significant changes occurred in several genes in the wnt and Notch signaling pathways, which are highly conserved and play critical roles in neuronal and glial differentiation. In the ubiquitin–proteasome pathway, proinflammatory cytokine mixtures induced upregulation of several genes, notably ubiquitin D (Ubd/FAT10), ubiquitin ligase and several proteasomal proteins. In agreement with microarray results, QRT-PCR showed marked upregulation of gene expression for Ubd with Th1 and M/M, for transglutaminase 2 with M/M, and for arginase 1 with Th2 cytokines. Expression of Ubd in the nervous system has not been previously reported. Both message and protein for Ubd are expressed in neurons, and upregulated by pro-inflammatory cytokines. Transglutaminase 2 has been implicated in neurodegenerative diseases, and proposed as a therapeutic target. Upregulation of arginase by Th2 cytokines could be potentially neuroprotective by decreasing NO generation and enhancing neurite outgrowth. Our analysis of changes in neuronal gene expression at the time of initial exposure to an abnormal cytokine milieu provides the opportunity to identify early changes that could be reversed to prevent later irreversible neuronal damage and death in multiple sclerosis and other CNS diseases.

1. Introduction

Loss of neurons and axonal damage are important in the pathogenesis of permanent disability in multiple sclerosis (MS) (Ferguson et al., 1997; Trapp et al., 1998; Wujek et al., 2002; Bjartmar and Trapp, 2003; Dutta and Trapp, 2010b), as in other neurodegenerative diseases. While some neuronal death in MS may result from damage to demyelinated axons (Waxman, 1998; Dutta and Trapp, 2010b), the possibility remains that inflammatory mediators, including cytokines, could also contribute to neuronal and axonal dysfunction and eventually cell death. This could occur by damage to axons in the white matter and/or damage to axons or neuronal cell bodies in the gray matter, even if the axon/neuron is not an antigen specific target of the immune process. Mediators of inflammation, particularly from microglia, members of the monocyte/macrophage

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doi:10.1016/j.jneuroim.2011.06.010

(M/M) cell lineage, are likely important in the pathogenesis of degenerative diseases of the central nervous system (CNS) including Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Akiyama et al., 2000; Minagar et al., 2004; Hensley et al., 2006; Bartels and Leenders, 2007). Some cytokines may also have direct or indirect protective effects in MS, AD, PD, ALS other CNS diseases.

In studies designed to examine the roles of cytokines in pathogenesis, repair and regeneration in MS, we previously analyzed the effects of mixtures of cytokines on early gene regulation by mixtures of CNS glial cells, employing gene array technology (Lisak et al., 2006, 2007, 2009). These cytokine mixtures were typical of Th1 and Th2 cells and proinflammatory M/M, so called M1 monocytes and microglia. The use of a mixture of cytokines rather than a single cytokine more closely represents the situation in the CNS in experimental and human diseases and allows analysis of gene regulation by glial cells and neurons without the confounding effect of gene regulation within the inflammatory cells themselves. Many of the changes in genes regulated in our *in vitro* experiments have also been demonstrated in CNS tissue from patients with MS (Woodroofe and Cuzner, 1993; Whitney et al., 1999; Lock et al., 2002; Mycko et al., 2003; Tajouri et al., 2003), including normal appearing white matter (NAWM) (Graumann et al., 2003). The hypothesis of our study was that neuronal gene expression would be differentially regulated *in vitro* by cytokine mixtures typical of Th1 and Th2 T cells and M/M.

2. Materials and methods

2.1. Neuronal cultures

Cultures highly enriched in neurons were isolated from 2 to 3 day old Sprague Dawley rat brain with minor modifications of the method of Eide and McMurray (2005), with the addition of 1 μg/ml nerve growth factor (NGF). Briefly, brains are rolled on moistened filter paper to remove meninges; tissue is minced and placed in dissociation medium containing 2 mg/ml papain, triturated with a 1 ml glass pipette, and incubated for 10 min at 37 °C., with 1 brain per 2 ml of medium. The tissue is suspended, and undissociated tissue allowed to settle for 5 min at ambient temperature. The cloudy supernatant is centrifuged for 10 min at 1000×*g*; the cell pellet is washed once in plating medium containing 10% rat serum, pelleted at 1000×*g* for 10 min, then suspended gently in 2.0 ml of plating medium per brain. Cells are plated on poly-L-lysine coated coverslips for immunocytochemistry and trypan blue counts, or on poly-L-lysine coated T25 flasks, 1/2 brain/flask, for RNA extraction. One day after initial plating, the medium is supplemented with 1 μg/ml NGF (Invitrogen, Carlsbad, CA), along with cytosine arabinoside (1×10^{-5} M) to inhibit proliferation of non-neural cells. After 5 days in culture, plating medium was removed and feeding medium added. Cells were treated with cytokines 8 days later (13 days in culture). Neural basal medium, DMEM, F-12 medium with glutamine and B27 supplement were from Invitrogen; papain was from Worthington Biochemical Corp., Lakewood NJ. For cytokine treatment, serum was removed, and each mixture of cytokines added to two T25 flasks for 6 h (early gene expression). Each set of two flasks was pooled to give one sample.

Cell types were characterized using antibodies to phenotypic markers: neurofilament heavy (NF-H) and anti-NeuN for neurons (Sternberger et al., 1982; Mullen et al., 1992); glial fibrillary acidic protein (GFAP) for astrocytes (Raff et al., 1978) from Chemicon International Inc, Temecula, CA; galactolipids (GalL) for oligodendrocytes (Raff et al., 1978; Ranscht et al., 1982) and ED1 for microglia (Dijkstra et al., 1985). The composition of each culture was quantitated by counting cells on coverslips following double label immunocytochemistry for NeuN and GFAP. The coverslips were from the same explants used to prepare flasks for the gene array experiments. Several random fields (a total of 200+ cells) were counted on duplicate coverslips and the % of a given glial type relative to neurons was calculated. Cultures were incubated with the different cytokine mixtures or additional medium (controls) for 6 h. Cytotoxicity was determined at 24 h using the criterion of trypan blue uptake, as we have done in the past with glial cell cultures (Lisak et al., 2006).

2.2. Cytokine mixtures

Th-1: Rat recombinant interleukin-2 (rrIL-2) and rat recombinant interferon γ (rrIFN-γ), (R&D Systems Inc, Minneapolis, MN); rat recombinant tumor necrosis factor (rrTNF-α) (BD PharMingen, San Diego, CA), and recombinant mouse granulocyte colony stimulating factor (rmG-CSF; Pepro Tech, Rocky Hill, N.J.).

M/M: rrTNF-α; (as above), rrIL-1α, rrIL-1β, rrIL-6, rrIL-12p40 (all R&D Systems).

Th2: rrIL-4, rrIL-5, rrIL-10 (R&D Systems), mouse G-CSF (as above), porcine transforming growth factor-β (TGF-β) (R&D Systems), currently viewed by some as a Th3 or Treg cytokine. All individual cytokines were used at a final concentration of 10 ng/ml.

Three sets of cultures were used for gene array analysis. In each experiment, cells were incubated with mixtures of Th1, M/M, Th2 cytokines or additional culture medium (control) for 6 h. The data represent 3 experiments with two pooled T25 flasks per condition in each experiment.

3. Microarray experiments

3.1. RNA analysis

RNA was extracted and gene expression was analyzed using Affymetrix REA 230 2.0 microarrays, with techniques we have employed in experiments with mixed glial cell cultures (Lisak et al., 2006, 2007, 2009). After 6 h of incubation with cytokine mixtures or additional medium, cultures were washed, cells were scraped with a small volume of TRIzol (Gibco BRL, Grand Island, NY) and frozen for subsequent extraction of mRNA and reverse transcription. The RNA was quantitated at $A_{260\ nm}$ and the quality was assessed at $A_{260\ nm}/A_{280\ nm}$. The 28S/18S ratio was assessed using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE). and was >1.7 for all samples. The REA 230 2.0 microarray chip contains over 30,000 transcripts, representing over 28,000 genes.

3.2. Data analysis

For initial analysis in these screening experiments we considered up or downregulation of 1.2 fold with p value of <0.05 as significant. This arbitrary cutoff includes more false positive results than a higher cutoff, but allows identification of potentially significant changes that can be verified or discarded based on qPCR results. We compared gene expression induced by cytokine mixtures to that in control cultures as well as comparing gene expression induced by the cytokines mixtures with one another. To determine which metabolic signaling pathways were regulated in the neuronal cultures by each of the three cytokine mixtures, data were analyzed using the KEGG data bases (Kyoto Encyclopedia of Genes and Genomes) in the DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/). We also analyzed the results by GO (Gene Ontogeny) and by the most up and down regulated genes for each of the cytokine mixtures.

3.3. Quantitative real time-polymerase chain reaction (QRT-PCR) expression analysis

To confirm results from the gene arrays, we analyzed regulation of several genes of interest employing QRT-PCR, including ubiquitin D (Ubd), presenilin 1, amyloid beta precursor protein, Notch 1, arginase 1, toll like receptor 2 (TLR 2), and transglutaminase 2. Expression of message was analyzed by QRT-PCR on an ABI 7500Fast System, using