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Expression Profiling following Traumatic Brain Injury: A Review

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Traumatic brain injury (TBI) elicits a complex sequence of putative autodestructive and neuroprotective cellular cascades. It is hypothesized that the genomic responses of cells in the injured brain serve as the basis for these cascades. Traditional methods for analyzing differential gene expression following brain trauma demonstrate that immediate early genes, cytokines, transcription factors, and neurotrophic factors can all participate in the brain's active and directed response to injury, and may do so concurrently. It is this complexity and multiplicity of interrelated molecular mechanisms that has demanded new methods for comprehensive and parallel evaluation of putative as well as novel gene targets. Recent advances in DNA microarray technology have enabled the simultaneous evaluation of thousands of genes and the subsequent generation of massive amounts of biological data relevant to CNS injury. This emerging technology can serve to further current knowledge regarding recognized molecular cascades as well as to identify novel molecular mechanisms that occur throughout the post-traumatic period. The elucidation of the complex alterations in gene expression underlying the pathological sequelae following TBI is of central importance in the design of future therapeutic agents.

KEY WORDS: Traumatic brain injury; microarray; immediate early genes; cytokines; neurotrophic factors; gene expression.

INTRODUCTION

The complex pathological sequelae following TBI have demanded a more complete understanding of the molecular events occurring during the post-traumatic period. Superimposed on the initial mechanical injury to the brain are delayed secondary changes that develop over a period of minutes to weeks or even months. It is believed that the majority of these changes result in perturbations of the normal homeostatic mechanisms that can activate neurotoxic or autodestructive molecular cascades. Proceeding simultaneously, however, are protective or reparative processes that can preserve neural connections and restore functionality. It is hypothesized that the foundation of these delayed pathologic and/or neuroprotective changes lies in a neuron's genomic response to the initial injury (1–3). Alterations in transcription of genes such as immediate early genes, cytokines, transcription factors and neurotrophins may determine a neuron's response to trauma and, subsequently, its role in either restoration of function or progression of cell loss. Therefore, the relationship between specific gene

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expression and regional cell dysfunction or death holds the key to understanding the pathophysiology underlying brain injury and its associated neurobehavioral sequelae. The elucidation of these activated molecular cascades is paramount to an identification of novel putative targets for neuroprotective therapies.

Previous technology has limited the number of genes that could be simultaneously analyzed. Recent advances in the sensitivity and reduced cost of DNA microarrays, though, have allowed for analysis of large numbers of genes (>8,000) during a single experiment (reviewed in Refs. 4 and 5). Parallel monitoring of thousands of genes has allowed for a more global view of the molecular mechanisms underlying head injury, switching the focus from the derangement of a single gene or gene family to alterations of the complex interactions between gene families. These new techniques have introduced a "paradigm shift" regarding our understanding of the complexity of post-traumatic molecular cascades and the multiplicity of their interrelated elements. Recently developed techniques in the isolation of messenger RNA (mRNA) now provide a means to analyze genomic changes from a single cell in the traumatically injured brain. Previous analyses of gene expression have been limited by the necessity for large amounts of RNA isolated from milligrams of freshly dissected tissues composed of heterogeneous cell types. The resulting expression profiles of particular genes were imprecise at best, especially in injured CNS tissue where a multitude of cell types have differing post-traumatic responses. Through the use of linear amplification methodology, the analysis of gene expression has been refined to encompass large families of genes expressed by a single cell (6–8). Reduction in the heterogeneity of cellular material holds the promise of accelerating both our understanding of the molecular cascades involved following head injury and the development of potential targets for future therapy.

Expression Profiling with Traditional Methods

Regardless of the advances in array technology experienced over the last several years, the majority of data regarding differential gene expression following TBI has been reported using traditional methods such as reverse transcription-polymerase chain reaction (RT-PCR), Northern blot (9), nuclease protection assay (NPA) (ribonuclease (10), S1 (11)), and subtractive hybridization (12). Although each technique has specific limitations, these methods do enable direct comparisons of expression levels among limited numbers of genes. For instance, RT-PCR is still considered by many to be the most sensitive technique for mRNA detection currently available (13). However, it's reliance on non-linear amplification weakens the quantification of the differential expression. On the other hand, NPAs are extremely sensitive in the quantitation of specific mRNAs from a complex mixture of total cellular RNA, but require a large amount of total RNA as starting material (14). Northern blot analysis still remains the standard for detection and quantitation of mRNA levels (15), despite its dependence on high quality, non-degraded RNA samples (e.g., RNA samples that are even slightly degraded can severely compromise the quality of the data and the ability to quantitate expression). Due to the disadvantages and limitations specific to each technique, direct comparison of results across these techniques can be problematic when trying to generate a global picture of transcriptional regulation following TBI.

A detailed description of the published studies analyzing gene expression in TBI conducted over the last decade is beyond the scope of this review. Therefore, we will focus on changes seen in transcription of immediate early genes, cytokines, transcription factors, and neurotrophin genes to illustrate how conventional expression profiling techniques have been applied in experimental models of TBI and how changes in these gene families have led to insight into the pathophysiology of brain trauma.

Immediate Early and Heat Shock Genes. Immediate early genes (IEGs) include proto-oncogenes of the c-*fos* and c-*jun* families (16,17), which, following protein dimerization, function as transcription factor complexes that bind specifically to consensus promoter sequences upstream of target genes (18). The fos protein forms heterodimers with various Jun proteins to form an active AP-1 complex (18). By binding to upstream modulating regions, these IEGs facilitate the initiation of transcription of other genes that further mediate the neuronal responses to brain injury. Some of the downstream gene targets whose differential expression is orchestrated by IEG binding include nerve growth factor (NGF) (19), amyloid precursor protein (APP) (20), and opioid precursor proteins (21), Moreover, the expression of some IEGs, particularly c-*jun* and c-*fos,* has also been associated with programmed cell death (22,23). Due to their ubiquitous nature, IEGs represent a potentially important interface between the initial (primary) responses and later pathological manifestations in TBI. In-depth analyses of a wide range of pathologic insults to the CNS have consistently revealed increases in IEGs at either the transcriptional or translational level. For example, the

prototypical IEG, c-*fos*, has been shown to be transiently increased in the cerebral cortex and hippocampus in rodent models of cerebral ischemia (30 to 90 min following middle cerebral artery occlusion) (24), in nuclei of granule cells in the rat dentate gyrus rapidly following seizure induction (25), and in the cortex and hippocampus following mechanical brain injury at both the protein (26) and mRNA level (27). Knowledge of the regulation of these genes and/or their potential targets may ultimately suggest new therapeutic approaches to the treatment of TBI.

The initial studies concerning the differential expression of IEGs following TBI were restricted to c-*fos* (28,29) (Table I). Using in situ hybridization, researchers detected a regionally specific increase in c-*fos* transcription as early as 1 hr post-injury (29,30). This increase was limited to the injured cortex following fluid percussion (FP) brain injury (28), and to bilateral cortical and hippocampal regions following controlled cortical impact (CCI) brain injury (29). Subsequent RT-PCR analysis in the ipsilateral cortex showed that this increase was detectable as early as 5 min following CCI brain injury and peaked at 1 hr post-injury (29). By 1 day post-injury, transcriptional over-expression had returned to pre-injury levels (28, 29). Raghupathi and colleagues also extended their earlier observations to the other IEGs required to form the active complex, c-*jun* and jun B (30). An increase of jun B expression was observed by in situ hybridization as early as 5 min post-injury and was maximal at 30 min post-injury. Similar to the previously reported expression of c-*fos* following FP brain injury, expression of jun B was localized bilaterally in the hippocampus and in the injured cortex. However, unlike jun B and c-*fos*, increases in c-*jun* mRNA expression were limited to the hippocampal dentate gyrus and persisted for greater than 6 hr post-injury (30).

Although the mechanism by which these IEGs are up-regulated is currently not known, it has been suggested that post-traumatic increases in intracellular free calcium may play a role (1,3). Increases in intracellular free calcium after TBI are largely mediated via the activation of voltage-sensitive channels and/or the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor (31). Intracellular ionic calcium, once increased, activates protein kinase C (PKC) and calmodulin kinase (CaMKII) which, in turn, activate the transcription factors serum response factor (SRF) and calcium/cAMP response element binding protein (CREB), respectively (17). Increases in cellular levels of SRF and CREB result in the transcription of fos and jun mRNAs (17). Although not in TBI, Sharp and colleagues demonstrated a direct link between calcium, IEG expression, and neural injury, using cultured cortical neurons and a variety of noxious stimuli (reviewed in Ref. 32).

The same pathologic conditions that induce IEG expression also appear to induce expression of the 72-kDa

Table I. A Tabular Representation of the Induction in mRNA Expression of Immediate Early Genes following Brain Injury

Gene	Injury model	Time	Region
c-*fos*	FP injury	5 min, 30 min, 2 hr	Cortex
		5 min, 30 min	Hippocampus
	CCI Injury	30 min, 1 hr, 3 hr	Cortex
		30 min, 1 hr, 3 hr	Hippocampus
	Cerebral ischemia	15 min, 30 min, 60 min, 90 min, 3 hr, 4 hr	Cortex
		30 min, 90 min	Hippocampus
	Cortical stab	15 min, 30 min, 60 min	Cortex, hippocampus
c-*jun*	FP injury	5 min, 6 hr	Hippocampus
junB	Cerebral ischemia	30 min, 60 min, 90 min, 4 hr	Cortex
		30 min, 90 min	Hippocampus
	FP injury	5 min, 2 hr	Cortex
		30 min, 2 hr	Hippocampus
hsp72	FP injury	2 hr, 6 hr, 12 hr	Cortex
	Cerebral ischemia	1 hr, 4 hr, 6 hr, 24 hr	Cortex
		2 hr, 6 hr, 12 hr	Hippocampus
	Cortical stab	2 hr, 12 hr	Cortex
	Seizures	6 hr, 12 hr	Hippocampus

Protein levels are not summarized here. All the brain regions represented are ipsilateral to the site of injury.

heat-shock protein (hsp72). For example, translation and transcription of hsp72 increases following ischemia (33–35), seizures (36), and forms of direct cortical trauma (surgical cuts) (37). Moreover, there are important correlations between the induction of c-*fos* and c-*jun* and the synthesis of stress proteins such as hsp70 (38). The heat shock protein gene family members are highly conserved and are named because of their discovery in cells exposed to heat-activated stress (39). These proteins function as chaperones to prevent the aggregation of incompletely folded peptides and facilitate the correct folding of proteins (39–41). However, they have also been reported to participate in a large number of other functions including dissociating clathrin baskets in the presence of ATP (a requirement for membrane recycling) (39), interacting with cellular tumor suppressor p53 (42), and protecting cultured neurons from glutamate toxicity (43).

Following FP brain injury in rats, an increase in hsp72 immunoreactivity was initially reported in the injured cortex that localized, primarily but not exclusively, in neurons (44). Subsequently, Lowenstein and co-workers used Northern blots to demonstrate that the level of hsp72 mRNA was increased in the injured cortex up to 12 hrs following TBI (45). Using in situ hybridization, a similar increase in hsp72 transcription was demonstrated as early as 2 hr post-injury in areas of the cortex immediately surrounding the site of maximal injury (28). This increase in hsp72 transcription was maintained up to 6 hr following injury, consistently localized to the cortex (deep cortex/white matter) (28). This differential expression was specific to hsp72, because the glucose-regulated proteins, grp78 and grp94, which share sequence homology with hsp72, were only mildly affected by TBI (45). The precise role of this hsp72 induction in response to TBI is still unknown and may simply reflect generalized stress response (34,46). However, it has also been hypothesized that up-regulation of hsp72 may serve a neuroprotective function (34,47).

Cytokines. Little doubt exists regarding the importance of inflammation in mediating delayed neuronal damage following CNS trauma. Infiltration of circulating immunocompetent cells into the brain parenchyma occurs almost immediately following trauma-induced opening of the blood-brain-barrier (BBB) (48,49). Entry of polymorphonuclear leukocytes (PMNs) into the injured brain is believed to mediate the local inflammatory response by releasing cytokines. Moreover, the entry of macrophages and/or microglia has been proposed as a key cellular event in the process of progressive tissue necrosis following brain injury (50). The cytokines most widely studied in the context of brain injury are the interleukins, such as IL-1β, IL-6, and tumor necrosis factor (TNFα) (1) (Table II). TNFα and IL-1 are produced in the brain in response to stimuli that alter glial homeostasis (51,52) and may function to induce activation and/or proliferation of astrocytes and microglia (53,54). Although induction of TNFα and IL-1 appears to be essential for cellular signaling within the brain to respond to an injury, a subsequent down-regulation of gene expression for these peptides may be critical for the continuation of repair. It is hypothesized that in the cytokine cascade, this down-regulation is mediated by a concomitant up-regulation in IL-6 (55). The study of the temporal pattern and cellular localization of the transcription of these cytokines has begun to address the importance of these immunological factors in mediating regional damage following brain trauma.

Young and coworkers demonstrated that patients with severe head injuries had significantly elevated levels of IL-1 and IL-6 in the cerebral spinal fluid (CSF) (56,57). Gourin and coworkers demonstrated that cultured human cerebral microvascular endothelial cells following percussive injury also released these cytokines (58). Similarly, cytokine proteins have also shown to be up-regulated at the translation level in several animal models following TBI (51,59,60). At the transcriptional level, Fan and colleagues reported that lateral FP brain injury in the rat increased the expression of IL-1β and TNFα mRNA in the injured cortex and hippocampus as early as 1 hr following injury (61,62). Moreover, TNFα mRNA was elevated in the hemisphere contralateral to the injury up to 1 hr post-trauma (62). However, because the differential expression in these studies was assessed using Northern blotting, nothing is known about the cell type responsible for the increased gene expression.

It has been hypothesized that activated microglia may, in part, be responsible for the production of IL-1 and IL-6 (51). Alternatively, evidence demonstrates that both IL-1β and TNFα mRNA are synthesized by peripheral immune cells and by neurons and glia in the

Table II. A Tabular Representation of the Induction in mRNA Expression of Cytokine Genes following Brain Injury

Gene	Injury model	Time	Region
IL-1β	FP injury	1 hr, 6 hr	Cortex, hippocampus
TNFα	FP injury	1 hr, 6 hr	Cortex, hippocampus

Protein levels are not summarized here. All the brain regions represented are ipsilateral to the site of injury.

CNS (1). The up-regulation of these cytokines following brain injury is suggestive of their role in mediating some portion of the pathophysiology of TBI. Both IL-1β and TNFα have been known to mediate the synthesis and release of potentially neurotoxic molecules such as arachidonic acid and its metabolites (63). Administration of an IL-1 receptor antagonist (IL-1ra) significantly decreased neuronal death following lateral FP brain injury in rats (64). Subsequently, Sanderson and coworkers (65) demonstrated that systemic administration of IL-1ra not only attenuated cell loss in several of the vulnerable regions including the hippocampal CA3 and hilar cells of the dentate gyrus but also improved cognitive and neurobehavioral motor function. Scherbel et al. (66) recently reported that mice deficient in TNF (TNF−/−) exhibited milder behavioral deficits than did wild type mice following CCI. However, TNF−/− mice did not recover during the chronic post-injury period when compared with brain injured wild-type mice, suggesting that gene expression for certain cytokines may be deleterious in the acute post-injury period but may participate in neuronal survival and repair in the chronic post-injury period by inducing synthesis of growth factors, stimulation of astrocytes proliferation, and inhibition of calcium currents (63). The area of cytokine gene expression following TBI remains deserving of continued interest as the potentially deleterious or paradoxically beneficial role of cytokine gene expression in the setting of CNS injury is not well understood.

Neurotrophic Factors. Neurotrophins are a class of structurally related neurotrophic factors (NTFs) that provide trophic support for neurons during development and adult life (67), promote neuronal survival in animal models of brain injury (68,69), and restore neuronal connections by promoting axonal outgrowth (70). This class of neurotrophins includes nerve growth factor (NGF) (71), brain-derived neurotrophic factor (BDNF) (72), and neurotrophin-3 (NT-3) (73). These NTFs mediate their cellular effects through tyrosine kinase receptors, namely trkA for NGF (74), trkB for BDNF (75), and trkC for NT-3 (76). All these NTFs are synthesized by neurons in the adult rat brain, particularly in the hippocampus, and are altered following brain injury. DeKosky and colleagues reported an increase in NGF transcription and translation in the acute post-traumatic period predominantly in astrocytes following brain injury in rat (77,78) (Table III). Others have demonstrated that this induction occurs as early as 1 hr following brain injury in the hippocampus (79). Up-regulation of NGF gene expression may serve to decrease the concentration of free radicals by inducing the expression of free radical scavengers such as glutathione peroxidase and catalase (80). CSF concentrations of NGF protein have been reported to increase following human head injury (81,82). BDNF mRNA expression was significantly increased by 3 hrs post-injury in rat hippocampus following FP brain injury (83). Oyesiku and coworkers subsequently confirmed these changes in gene expression in a rat model of severe brain injury (84).

Table III. A Tabular Representation of the Induction in mRNA Expression of Neurotrophic Factors following Brain Injury

Gene	Injury model	Time	Region
NGF	CCI injury	24 hr	Cortex
		1 hr, 3 hr, 5 hr	Hippocampus
BDNF	FP injury	3 hr, 6 hr, 24 hr	Hippocampus
	CCI injury	12 hr	Cortex

Protein levels are not summarized here. All the brain regions represented are ipsilateral to the site of injury.

Up-regulation of NTF mRNA and protein after TBI is believed to represent an endogenous neuroprotective response. This hypothesis is supported by experimental studies in which these factors were pharmacologically administered following brain injury. Intraparenchymal administration of NGF has been shown to attenuate cognitive deficits following rat FP brain injury (85) and CCI brain injury (86). Central administration of NGF was subsequently shown to reduce the extent of apoptotic cell death in septal cholinergic neurons following experimental brain injury (87). Nonetheless, the association of increased NTF mRNA expression following brain injury with "improved" outcome by facilitating neuronal survival/repair and inducing the sprouting of neurites to re-establish functional connections is still not fully accepted.

Expression Profiling with Arrays

Historically, analysis of expression of multiple genes using the traditional methods listed previously was laborious due to the small number of genes each assay could simultaneously evaluate. Recently, DNA microarrays have emerged as the method of choice for the analyses of differential regulation following brain injury due to their ability to analyze simultaneously the expression of thousands of mRNAs. Microarrays provide the ability to not only corroborate previous differential expression studies but also exponentially increase the available data regarding alterations in gene expression. Moreover, because arrays allow for

analyses of a large numbers of currently uncharacterized genes, they also have the potential to increase substantially the speed at which novel mediators of post-traumatic cascades are identified. In hopes of advancing these aims, several researchers have utilized various methodologies to extract mRNA from brain-injured tissue and use it to probe both low- and high-density arrays.

One of the first studies utilizing this technology in TBI used the antisense RNA (aRNA) technique to isolate mRNA from an organotypic brain slice culture following mechanical stretch injury (88). The aRNA technique (7) was used to generate sufficient mRNA to probe an array while maintaining a linear rate of amplification. The arrays employed by Morrison and coworkers were nitrocellulose-based macroarrays incorporating 24 different clones including several genes involved in apoptosis, intracellular Ca^{2+} signaling, trophic factors signaling, and intercellular signaling. With the use of multiple time points, the authors were able to demonstrate a transient and simultaneous decrease at 6 hr post-injury in the expression of both $CamKII_{\alpha}$ and CREB with a return to baseline levels by 48 hr. Similarly, other genes, such as bcl-2, GAD65, $PKC_{1\beta}$, and ubiquitin, were differentially down-regulated during the first 24 hr following injury but returned back to baseline by 48 hr. Conversely, the expression of other genes, such as NGF, remained elevated at 24 hr and returned to baseline at 48 hr. With the use of arrays to simultaneously analyze 24 separate genes, the authors were able to demonstrate how the traumatized brain mounts an active genomic response by up- and down-regulating the expression of certain genes during the acute post-traumatic period. Because the expression of several other genes did not change, the observed differential expression appears to reflect a specific and directed response to trauma by selective activation or inactivation of molecular cascades.

Recently, Matzilevich and coworkers used high-density microarrays incorporating over 8,000 genes, many of which had not been previously implicated in TBI pathophysiology (89). The authors pooled the mRNA from the hippocampus ipsilateral to the injury from several brain-injured rats at 3 hr or 24 hr following CCI. Approximately 6% of the genes analyzed at either time point were differentially regulated in response to injury. Specific genes up- or down-regulated at 3 hr were different than those at 24 hr. Moreover, after having assigned each of the differentially regulated genes to a functional class (cell cycle, metabolism, NO and ROS metabolism, inflammation-related, receptors, signal transduction, cytoskeletal proteins, growth factors, neuropeptides, membrane proteins, channels and transporters, and transcription/translation) based on their reported or suggested function, the authors used cluster analysis (90) to determine similarities based on expression profiles. The changes in gene expression reported provide insight into the general progression of events that occur following TBI. For instance, transcription of genes encoding for growth factors, and proteins involved in glucose and reactive oxygen species metabolism and inflammation was increased early after brain injury and returned to baseline levels by 24 hr. The authors hypothesized that these may represent both intrinsic and extrinsic survival strategies aimed at protecting individual cells or the brain as a whole.

One common constraint in the above studies is that the total pool of mRNA studied was extracted from whole brain sections, each containing a heterogeneous population of cells. The complexity of this multicellular sample rests on the fact that various cell types mount different responses to the same induced injury, and the reported data likely dilute each individual cell's contribution. For instance, understanding the molecular mechanisms underlying the delayed neuronal death in the hippocampus following TBI would be complicated using mRNA harvested from the whole structure, in which the specificity of a neuron's contribution to the changes in differential expression would be lost. Therefore, it would be advantageous to analyze the mRNA from only specific cells of interest. Accordingly, O'Dell and colleagues used two rounds of the aRNA protocol to linearly amplify a pool of mRNAs from a single damaged neuron from the cortex of a brain-injured rat (91). The authors used terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) stain along with cellular morphology as a marker for apoptotic cell damage and adapted the aRNA protocol accordingly for use in histologically fixed tissue. The mRNAs obtained from single neurons at either 12 or 24 hr after TBI were analyzed using a nylon array with 31 candidate genes. Phenotypically matched TUNEL-positive cells from the cortex of brain-injured rats had unique expression profiles at 12 hr compared to 24 hr after injury. At 12 hr post-injury, statistically significant decreases in genes such as CREB, NFG, trkB, and bax were observed, which returned to baseline by 24 hr. Whether these reported differences in transcription are due to two separate co-existing molecular cascades or if they are two distinct points along a continuum of cell death is not currently understood. However, this increased insight into the individual neuron's molecular response to TBI using expression profiling can aid in

our understanding of the complex relationships among different cells and cell types following brain injury.

CONCLUSION

Despite great strides in understanding of the underlying pathobiology involved following brain injury, the complexity of the disease and the multiplicity of its interrelated elements are yet to be fully appreciated. Through the use of a number of experimental animal models, the regional and temporal patterns of cellular and molecular responses to TBI are being mapped. Recent data suggest that a number of potentially divergent cascades are induced by brain injury. Ample evidence suggests that the caspase cascade, whether activated or propagated by IEGs, plays a major role in mediating apoptotic cell death, which is regarded as a neuropathological hallmark of the acute post-traumatic period. The inhibition of these putative deleterious cascades via the use of specific inhibitors has proven to ameliorate the post-traumatic cognitive dysfunction. Conversely, also activated during the post-traumatic period are putative reparative mechanisms including up-regulation of gene expression for a variety of neurotrophic factors. Neurotrophins are synthesized by neurons and may function to restore neuronal connections by promoting axonal outgrowth in the days to weeks after brain trauma. Subsequent administration of these factors following TBI has provided encouraging results with respect to the attenuation of either the neurobehavioral deficits or cell loss associated with TBI.

Taken together, these findings highlight the complexity and heterogeneity of the pathologic and molecular responses to TBL. The understanding of these underlying pathways using expression profiling is essential to the formulation of a rational approach to treatment design.

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