Spatial and temporal gene expression profiling of the contused rat spinal cord

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Abstract

Microarray technology was used to examine gene expression changes following contusive injury of the adult rat spinal cord. To obtain a global understanding of the changes triggered by the injury, differential gene expression was examined spatially, using tissue samples from the epicenter of injury as well as 1 cm rostral and 1 cm caudal to the epicenter, and temporally, at 3 h, 24 h, 7 days, and 35 days post-injury. To filter out gene expression changes that were due to the laminectomy, samples of contused tissue were compared to laminectomy-only controls. We took advantage of four different, complementary methods of data analysis to detect differentially expressed genes. We have identified functional groups of genes that are differentially regulated in our model, including those associated with apoptosis, cell cycle, inflammation, and cholesterol metabolism. Our analysis has led to the identification of novel potential therapeutic targets within each group of genes that is discussed.
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Introduction

A better understanding of events occurring in the spinal cord after injury is essential to identify ways to limit secondary damage, promote axonal regeneration, and ultimately improve functional outcome. Many studies have investigated changes at the RNA and protein levels after spinal cord injury (SCI) but have been of limited scope, focusing only on a few genes of interest (Bartholdi and Schwab, 1997; Bartholdi et al., 1997; Grossman et al., 1999, 2000; Hayashi et al., 2000; Mautes and Noble, 2000; Mautes et al., 1998; McTigue et al., 1998; Yakovlev and Faden, 1994). The introduction of microarray technology, where thousands of genes can be studied in parallel, permits a broad assessment of gene changes after SCI.

Several studies have examined differential gene expression after contusive SCI using cDNA microarrays (Tachibana et al., 2002) or oligonucleotide-based arrays (Affymetrix chips; Carmel et al., 2001; Song et al., 2001). These studies examined acute gene expression changes and pooled RNA from multiple animals (see Table 1 for experimental parameters used in each study). Results of these early efforts showed an initial upregulation of genes for transcription factors, immediate early genes, heat shock proteins, and pro-inflammatory genes, whereas some neurotransmitter receptors and transporters, ion channels, kinases, and structural proteins were downregulated. Taken together, the results suggest that genes involved in repair are upregulated in an attempt to stabilize the injured spinal cord.

A recent paper by Di Giovanni et al. (2003) made many improvements over earlier studies by assessing gene changes after SCI in a more comprehensive fashion. This

Table 1
Characteristics of selected gene-expression studies of contusive spinal injury

Study	Injury site	Sex of rats	Tissue examined	Time points	Pooled RNA?	Analysis	Verification
Carmel et al. (2001)	T9–10	M/F	epicenter, distal	6, 12, 24, 48 h	yes	Affymetrix, GeneCluster software	Q-PCR
Song et al. (2001)	T9	F	epicenter	3, 24 h	yes	Affymetrix software	RT-PCR
Tachibana et al. (2002)	T12	M	epicenter	24 h	yes	ImageQuant	RT-PCR
Di Giovanni et al. (2003)	T8–9	M	epicenter	30 min, 4 h, 24 h, 7 days	no	Affymetrix, statistics, GeneSpring	RT-PCR, immunoblotting, immunocytochem
Present study	T8	F	epicenter, rostral, caudal	3, 24 h, 7, 35 days	no	Affymetrix, dChip, Naef, Drop	Q-PCR

study was the first to include a post-injury time point beyond 48 h. Furthermore, rather than merely generating gene lists, an in-depth statistical analysis combined with temporal and functional clustering produced comprehensive profiles of classes of differentially expressed genes, changes that were confirmed by multiple verification methods.

In the present paper, we made several additional advances to broaden our understanding of SCI-induced gene expression changes. First, we examined multiple regions of the spinal cord. Rather than targeting only the epicenter, we also examined areas rostral and caudal to the epicenter to elucidate events that may be occurring distal to the damage. Second, we examined changes at multiple time points: 3 h, 24 h, 7 days, and 35 days after injury, with the latter time point being added because earlier studies did not consider long-term gene expression changes. Third, unlike some prior studies, we did not pool RNA from multiple animals. Fourth, we filtered out gene expression changes that might be due to the laminectomy by including laminectomy-only control animals. Finally, we utilized multiple analysis methods (further explained in Materials and methods) to increase confidence in our candidate genes. From that pool of genes we generated profiles of expression changes; in this paper, we discuss the potential influence of these changes on major post-injury events, such as apoptosis, inflammation, and synaptic plasticity.

Materials and methods

Spinal cord injury and dissection

The spinal cords of 12 female Fischer 344 rats (three animals per time point; 165–200 g) were contused at T8, using the Ohio State device (Stokes, 1992). Rats were anesthetized with a mixture of ketamine (0.8 mg/kg, ip) and xylazine (0.5 mg/kg, ip) and laminectomies were performed, leaving the dura undisturbed. The T7 and T9 spinal processes were clamped in a spinal frame, and

contusions were made by rapidly displacing the cord 1.0 mm (moderate injury). Muscle layers were then sutured and skin layers closed with wound clips. Laminectomies without a contusion were performed in another 12 female Fisher rats (three animals per time point). Laminectomy-only animals as well as three intact, anesthetized controls underwent the same postoperative treatment as the contused animals. Baytril® (enrofloxacin, Bayer, Shawnee Mission, KS; 0.03 ml) and Ringer's solution (5 ml) were administered subcutaneously, and the rats were placed in warmed cages to recover from anesthesia. Postoperatively, rats were carefully monitored; Baytril® was administered twice daily for the first post-injury week and once daily for the second. Manual bladder expression was performed twice a day for the first week and once daily thereafter until the return of normal bladder function (usually 10–14 days). Fluids and a highly nutritious, palatable supplement (Nutrical, Evsco Pharmaceuticals, Buena, NJ; 5 ml) were administered daily for the first 3 days. Rats surviving for 7 or 35 days were observed for appropriate return of stepping. At 7 days, all rats were moving all joints of both hindlimbs, and at 35 days all were capable of locomotion. At each postoperative time point examined, rats were overdosed with a mixture of ketamine (80 mg/kg), xylazine (4 mg/kg), and acepromazine (0.75 mg/kg, ip) and decapitated. The spinal cord was rapidly dissected (≤6 min), the epicenter of injury was located, and samples were taken and placed in polypropylene tubes on dry ice. Because the laminectomy-only animals did not have a lesion, the "epicenter" was marked on the dura with ink at the level of the laminectomy. Intact animals had no injury or laminectomy, so the T8 process was identified and removed, and the cord below was marked as "epicenter." Otherwise, segments were cut and frozen in a manner identical to those of the contusions.

Experimental design

Four different postoperative time points (3 h, 24 h, 7 days, and 35 days after injury) and three separate injury conditions (Intact, Laminectomy, and Contusion) were ex-

amined in this study. At each time point, three rats from the Laminectomy and Contusion conditions were sacrificed. Three separate tissue samples were then taken from each animal as follows. First, a 1-cm segment encompassing the injury epicenter ("Epicenter") was taken. Next, 1-cm segments of cord immediately rostral and immediately caudal to the Epicenter were cut away and discarded. The next adjacent rostral and caudal 1-cm segments were then taken as samples ("Rostral" and "Caudal," respectively; Fig. 1). It should be noted that, in injured animals, the "Epicenter" sample contained not only the center of the lesion (where the damage was most severe) but also the entire lesion area, including inflammatory cells that invaded the injured tissue. Because contusion lesions enlarge over time (Grossman et al., 2001), in animals sacrificed hours after injury the 1-cm Epicenter sample also contained undamaged tissue at either end of the lesion; by contrast, in animals sacrificed days after injury, the lesion comprised areas of secondary damage which extended throughout the Epicenter sample.

RNA extraction and microarray procedures

Total RNA was extracted from each sample individually (Rostral, Epicenter, Caudal) using TRIzol reagent (Invitrogen, Carlsbad, CA). The quality of the RNA was assessed by spectroscopy—A260/A280 ratio in Tris–EDTA (pH 8.0)—and ranged between 2 and 2.1. Agarose gels (1%) were run to verify that RNA was not degraded. Total RNA (10 μg) was used to generate first-strand cDNA by using a T7-linked-$(dT)_{24}$ primer. After second-strand synthesis, the double-stranded cDNA was purified by phase-lock gels, extracted by phenol/chloroform, ethanol precipitated, and then used for in vitro transcription using the ENZO BioArray High-Yield RNA transcript labeling kit (ENZO Farmingdale, NY). The labeled cRNA was purified using the RNeasy kit (Qiagen, Valencia, CA) and was fragmented to approximately 25 base sequences by incubation at 94°C.

Each fragmented, biotin-labeled cRNA sample (30 μg) was hybridized to an Affymetrix rat RG-U34A chip for 16
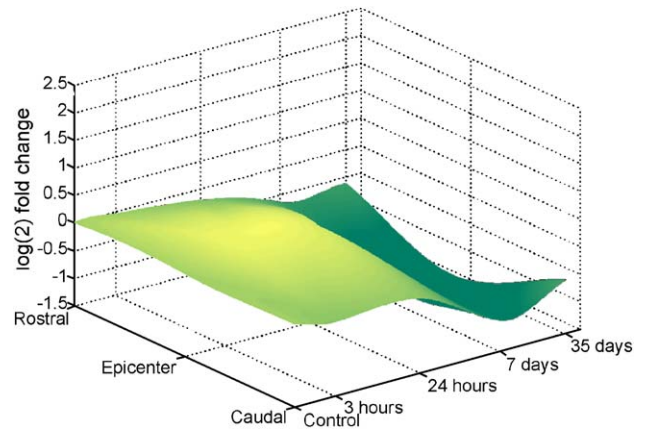
Fig. 2. Spatiotemporal profile of expression of the gene coding for HMG CoA reductase. This gene is robustly downregulated in all three areas sampled, rostral, epicenter, and caudal at 35 days post-injury. This and all other spatiotemporal gene expression profiles depicted in this report show $\log_2$ fold change on the z-axis, with the time point on the x-axis and region on the y-axis.

hours and rotated at 13 rpm at 50°C. Bacterial controls were spiked in. The chips were washed and stained using a streptavidin–phycoerythrin conjugate (Molecular Probes, Eugene, OR), a goat IgG antibody, and then a second streptavidin–phycoerythrin stain and were scanned on an Affymetrix GeneChip scanner. After scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches. Chips had to meet the following criteria to be included in the data analysis: the number of probe pairs called present had to be greater than or equal to 30%, all spiked-in bacterial controls had to be called present, background had to be <200, the number of outliers had to be <500, and the 3'/5' ratio of GAPDH and actin had to be <2.

Data analysis

Data derived from GeneChips have two potential sources of error: experimental and analytical. While experimental error can be limited by higher quality samples and standardized hybridization procedures, analytical error is difficult to control. The standard goal of minimizing both false positives and false negatives is compromised by the assumption-laden tools used for analysis. These assumptions also contribute to a high degree of variance between different analysis methods, presenting an additional challenge to the interpretation of resultant gene changes.

There are several tools available to analyze GeneChip data. Because those appropriate for use with Affymetrix chips are highly variable in their performance, we chose four of the available methods. This multiple-method approach (D'Amour and Gage, 2003) improved analytical confidence for several reasons. First, if several methods determined the same gene to be changed, it is unlikely that this change occurred simply as a result of an analytical
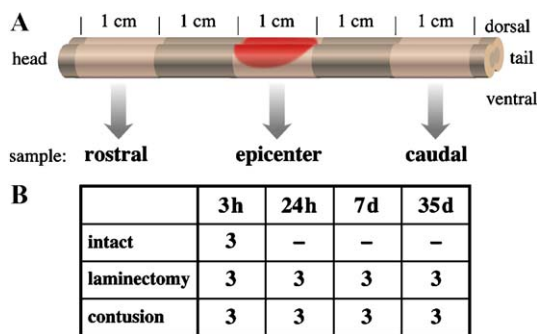

Fig. 1. (A) Location of samples taken from spinal cord tissue with respect to the epicenter of injury. (B) Experimental design. The number inside each cell denotes the number of chips processed for each condition.

assumption inherent to a single method. Second, false negatives that arose from intrinsic analytical assumptions in one method were unlikely to be repeated in each of the other methods. Third, the approach offered a systematic way of monitoring the potential for a gene to be an analytical false positive.

The chips were analyzed using four different complementary analytical tools (see Table 2). Data were preprocessed (*.dat files to *.cel files) using Affymetrix Microarray Analysis Suite (MAS) 4.0. Data were subsequently analyzed using the MAS 4.0 algorithms, dChip 1.3 PM-MM (Li and Wong, 2001), Drop Method (Aimone and Gage, 2004), and the algorithms of Felix Naef (Naef et al., 2002). dChip approximates expression values by modeling the whole body of chip data to an ideal system, Drop determines the significance of change between the triplicate groups statistically with minimal assumptions, and the Naef method calculates statistical changes using only the Perfect Match probes. MAS 4.0 uses a unique empirical approach to analysis which was also used since it allows comparison of our results to previous SCI GeneChip studies which used only MAS 4.0, although it is now rarely used in current GeneChip experiments. By combining the triplicate group and individual chip statistical approaches with model-based and empirically based methods, we are able to obtain an effective characterization of differential gene expression without the risk of significant changes being masked by inappropriate mathematical or statistical assumptions.

For each region, Contusion chips at each time point were compared to Intact and Laminectomy chips at the same time point, yielding 24 different triplicate group comparisons, from which lists of changed genes were generated. Each group had three chips, so each comparison had a three-by-three design, with a possibility of nine pairwise comparisons for each group. For statistical significance, we imposed a statistical threshold of $P < 0.05$ ($c > 95\%$ in Drop) in all methods except MAS, which makes an empirical difference call between probe sets on a pair of chips. Each method makes this statistical assessment differently. Drop makes a confidence call (c value) based on behavior of the individual probes

between triplicate groups, Naef measures the statistical difference of the probe set between two chips, and dChip calculates a t test significance of probe set values (thetas) between triplicate groups. In MAS 4.0, an "Increase" or "Decrease" call (or "Marginal") was required for each pair of chips.

Although P values are sufficient for most biological analyses, if used alone in microarray experiments they have been shown to lead to substantial numbers of false positives (Nadon et al., 2002). Therefore, we imposed an additional fold change restriction on the methods that perform whole probe set comparisons (MAS 4.0, dChip 1.3, and Felix Naef) to both reduce the effect of parallel statistics as well as add an addition filter of biological relevance. The whole probe set expression for each chip in a triplicate group must have demonstrated at least a 1.2-fold change vs. each other chip in the comparison group. Since the Drop Method performed statistical analysis on unaltered individual probe measurements before probe set consolidation, no fold change restriction was needed. The fold change restriction also provided additional confidence in the resultant lists by allowing for an unseen biological variability of up to 20% within this injury model because of the small sample sizes (see Table 2 for a complete list of filtering criteria).

Three-dimensional plots

Three-dimensional plots were generated using MATLAB (The MathWorks, Inc.) to enhance visualization of differential gene expression across tissue sample site and time after injury. The x-axis represents a logged time from injury (no injury, 3 h, 24 h, 7 days, 35 days). The y-axis was set as the distance along the spinal cord from which the sample was taken. The z-axis values and colors were set by taking the $\log_2$ ratio of contusion to laminectomy (or control) at each data point from dChip. The intermittent values were estimated by the default MATLAB algorithms to form a smooth transition between data points.

GeneSpring

The expression values generated by processing with dChip were used for further analysis in GeneSpring (Silicon Genetics). Upon data entry into GeneSpring, expression values below 0 were set to 0. Each probe set was divided by the median of its measurements in all samples. If the median of the raw values was below 10, then each measurement for that probe set was divided by 10. No per chip normalization was applied to the samples in GeneSpring as the data from each chip had been previously normalized to the median intensity chip in dChip.

Lists of probe sets that were significantly changed were generated and classified as being changed in either one or

Table 2
Criteria used for filtering gene lists

Method	Filtering criteria
Affymetrix 4.0	(1) fold change (FC) >1.2 for all pairwise comparisons
	(2) diff call (I/MI/MD/D) for all pairwise comparisons
dChip 1.3	(1) P value <0.05 for Group A vs. Group B
	(2) FC > 1.2 (LCB)[a] for all pairwise comparisons
Drop	(1) c[b] > 95% for Group A vs. Group B
Naef	(1) $P < 0.05$ for all pairwise comparisons
	(2) FC > 1.2 for all pairwise comparisons
	(3) $\log_2/SD > 1.25$ for all pairwise comparisons

[a] Lower confidence boundary.
[b] Confidence interval.

more methods or as changed in three or more methods. These lists were used in GeneSpring for filtering against lists of genes of interest. GeneSpring was used to identify probe sets with similar expression patterns to a gene of interest using the "find like gene" function.

K-means clustering was used to separate lists of probe sets of interest into groups of probes sets with similar expression patterns. For consistency, the "find best K means" script within GeneSpring was used. This script tries a K-means classification with 3, 5, 8, and 15 clusters and chooses the one with the highest explained variability.

Pathway analysis

As a complement to the unbiased mathematical approach that clustering provides, we inspected our results for patterns of changes suspected to be involved in SCI and subsequent processes. Biological and functional relationships between genes measured were examined using GenMAPP software (http://www.genmapp.org), which provided a mechanism to view significant changes and trends within known pathways ranging from metabolism to signal transduction as well as within functional groups such as channel classes and receptor types. The majority of the biological pathways evaluated were provided by Gen-MAPP, although in some cases these were customized to include additional relationships that are discussed in the literature.

Real-time quantitative PCR (Q-PCR)

Expression changes of genes of interest were verified by quantitative RT-PCR (Q-PCR). cDNA was prepared from RNA samples used for GeneChip analysis, and reverse transcription was performed using Superscript First-strand cDNA Synthesis System (catalogue number 119/04-018; Invitrogen). Primer Express v1.5 software was used to design oligonucleotide primers (Applied Biosystems, Foster City, CA) (see Table 3 for sequences of primers) which were constructed by Allele Biotechnology

Table 4
Q-PCR results

	Q-PCR fold change	GeneChip fold change	Time point and region examined
Ca^{2+}-ATPase isoform 2	−9.8	−2.0	35 days, epicenter
Ca^{2+}-sensitive calmodulin-sensitive	−46.8	−1.8	24 h, epicenter
Caspase 7	1.3	6.5	35 days, rostral
Cathepsin B	−1.4	2.0	35 days, epicenter
Cathepsin D	1.2	3.5	35 days, epicenter
Cathepsin L	8.8	1.6	35 days, epicenter
Cystatin C	−20.8	−1.4	24 h, epicenter
GAD 67	−10.0	−2.3	35 days, epicenter
HMG CoA reductase	−13.5	−3.1	35 days, epicenter
HMG CoA reductase	−4.5	−1.5	35 days, caudal
HMG CoA reductase	−1.4	−1.9	35 days, rostral
ICAM	7.4	2.3	3 h, epicenter
IL-6	9.8	40.6	24 h, epicenter
Metallothionein I and II	2.3	1.6	3 h, epicenter
p-Selectin	8.3	2.1	3 h, epicenter
Scya 2	10.0	12.2	24 h, epicenter
Syndecan 4	2.2	1.9	3 h, epicenter
VCAM	4.1	1.7	3 h, epicenter
VEGFa	1.7	2.0	24 h, epicenter[a]

All comparisons were made between contusion and laminectomy samples.
[a] Lesion compared with Epicenter sample from intact control.

Table 3
Primer sequences used for quantitative real-time PCR (Q-PCR)

Candidate gene	5′ primer	3′ primer
18s rRNA	CTTAGTTGGTGGAGCGATTTGTC	AGAGTCTCGTTCGTTATCGGAATT
Ca^{2+}-ATPase isoform 2	CGAGAGCTTCCGCATGTACA	GCAGCACTTTTTGAGCACAATCT
Ca^{2+}-sensitive calmodulin-sensitive	TGGGCCTCTCCTTCTATGCA	ACACATTCCCACAGGCTTCACT
Caspase 7	CCACTTACCTGTACCGTATGGACTT	TGATGATACATTTGCCCATCTTCT
Cathepsin B	GCAGGCTGGACGCAACTT	AGCTTCTTCAGATAGCTTATGTCAACA
Cathepsin D	ACGGAAGTGGGAGGCTCTGT	GGTTATGGGACCTTTAAGGATCAG
Cathepsin L	TGGAGAGAAAAGGGTTGTGTGA	AGAACCACACTGGCCCTGAT
Cystatin C	AGCCCCATCTGATGAGGAA	CAGGGCACGCTGTAGATCTG
GAD 67	CAGAAAACTGGGCCTGAAGATC	AGGCTATTGGTCCTTTGCAAGA
HMG CoA reductase	GGTGCATCGCCATCCTGTA	GCTGACGCAGGTTCTGGAA
ICAM	GACGAACTATCGAGTGGACACAACT	CAATGTCGCTCAGCTTGAAGAG
IL-6	TGTTCTCAGGGAGATCTTGGAAA	CAGAATTGCCATTGCACAACTC
Metallothionein I and II	GCTCCTGTGCCACAGATG	CAGGAACAGCAGTTTTCTTGCA
P-selectin	ACAGACTTAGTGGCCATCCAGAA	GTCATTGAGGTGAGCGATTTCA
Scya 2	CCAATAAGGAATGGGTCCAGAAG	CACTTGGTTCTGGTCCAGTTTTC
Syndecan 4	TTTCACCCTTGGTGCCACTAG	GCCAGGCTGGGCATTCT
VCAM	TCGGAGCCTCAACGGTACTT	TTTAAAAGCTTGAGAGACTGCAAACA
VEGFa	GACGTCTACCAGCGCAGCTAT	GATGTCCACCAGGGTCTCAATT

and Pharmaceuticals, Inc. (San Diego, CA). Q-PCR was performed with 3.33 ng of cDNA, SYBR Green Master Mix (Applied Biosystems), and 0.2 μM of each primer in a 25-μl reaction. All reactions were performed in triplicate using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Data analysis was performed according to the protocol provided by Applied Biosystems using Sequence Detector Systems v1.7 software. A mixture of intact rat brain and spinal cord cDNA was used to create standard curves in most cases. Some genes were not expressed in a high enough level in this mixture of standard curve cDNA (i.e., ICAM, VEGF, scya2); therefore, a mixture of laminectomy and lesioned cDNA (from the 24-h Epicenter samples or from the Rostral 35-day Epicenter samples) was used to generate the standard curve for these genes. Expression of each gene was calculated based on the standard curve for a given primer set. The relative amount of calculated message was normalized to the level of a control gene (18s rRNA). The Q-PCR results are summarized in Table 4.

Results

Concurrent use of the four analysis methods described in Materials and methods generated lists of "hits" with probable true expression changes. These lists then required further analysis to extract interesting genes and gene interrelationships. Complete lists of genes demonstrating significant expression changes for each region at each time point can be viewed at http://www.ChristopherReeve.org/microarray. We used a variety of techniques to process our lists, including clustering, pathway mapping, and three-dimensional displays (described in Materials and methods). This secondary processing yielded many intriguing profiles of gene activity after SCI, the most interesting examples of which are highlighted below. We selected specific genes from pathways of interest and verified their gene expression changes using Q-PCR. In addition to Q-PCR confirmation and corroboration of results from literature, the co-regulation of genes within the same regulatory pathways can serve as additional support for real expression changes.

Downregulation of genes coding for enzymes involved in cholesterol biosynthesis

Our microarray data revealed, and Q-PCR results confirmed, that expression of the gene coding for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) was downregulated 35 days after injury in the Epicenter, as well as in the Rostral and Caudal samples (see Fig. 2). Our data also revealed a chronic decrease in the expression of other genes coding for enzymes involved in cholesterol synthesis, including isopentenyl pyrophosphate isomerase, dimethyl-trans-transferase, squalene synthetase, and squalene monooxygenase.

Expression of genes coding for proteins involved in neurotransmission

The transcript for tyrosine 3 monooxygenase/tryptophan 5 monooxygenase activating protein, which acts in association with calcium/calmodulin-dependent protein kinase II (Cam2k) to activate enzymes that are necessary for the synthesis of dopamine, norepinephrine, and epinephrine (Yamauchi et al., 1981), was less abundant in the Epicenter as well as in Rostral and Caudal segments after 35 days. Cam2k was also downregulated at the injury epicenter, as well as phenylethanolamine *N*-methyltransferase, the key enzyme in converting norepinephrine to epinephrine (see Fig. 3 and Table 5 for details). Also, less of the transcript for glutamate decarboxylase (GAD 67), the synthetic enzyme for converting glutamate to GABA, was detectable in the Epicenter at 35 days, a result confirmed by subsequent Q-PCR analysis (see Table 4). Our analysis also showed changes in genes that are related to neurotransmitter release, transport, and uptake. The gene for glial glycine transporter 1 was downregulated in the Epicenter at 24 h and in the Caudal segment at the 3-h and 7-day time points, while expression of the gene coding for the GABA transporter was decreased in the Epicenter. In addition, the genes for monoamine oxidase B and acetylcholinesterase were shown to be downregulated after injury (see Table 5 for details). Changes were also found in the expression of genes involved in formation and docking of synaptic vesicles, including synaptophysin, synapsin I, synapsin II, synaptic glycoprotein SC2, synaptobrevin 2, synaptic vesicle glycoprotein 2a, syntaxin 2, synaptojanin 1, and SNAP 25A. Interestingly, the transcript of the gene for synaptic vesicle glycoprotein 2b was less abundant until 7 days after injury, after which point it was increased in the Epicenter sample.

Genes related to calcium signaling

Expression of the gene coding for the Ca^{2+} pump, Ca^{2+}-ATPase, was decreased with injury. Similarly, other Ca^{2+}-related genes were downregulated, including several voltage-gated (L-type) Ca^{2+} channels, Ca^{2+}-permeable ionotropic glutamate (AMPA) channels, and other Ca^{2+}-dependent intracellular signaling molecules such as G protein G0 (alpha subunit) and phospholipase C (see Fig. 3 and Table 5).

Genes associated with inflammation, immune cell recruitment, and extravasation

The present data indicate that IL-1α was increased in the Epicenter sample at 3 h post-lesion, whereas IL-1β was increased at 3 and 24 h post-SCI (see Figs. 4 and 5). The only significant change in anti-inflammatory factors that we observed was an upregulation of IL-1RII within the injury epicenter at 24 h post-injury. Scya2, or monocyte chemoattractant protein-1 (MCP-1), is a complement cascade chemokine that acts on monocytes, T cells, and NK cells.

Fig. 3. Several important cellular events related to synaptic plasticity are disrupted by SCI. Genes for enzymes related to neurotransmitter biosynthesis are disrupted (e.g., GAD 67), as are synaptic vesicle proteins (e.g., SNAP 25A). In addition to disruptions in genes related to neurotransmitter transport and degradation, genes involved both pre- and postsynaptically in calcium signaling are disrupted (e.g., L-type Ca^{2+} channels and Ca^{2+}-ATPase). The increase in intracellular Ca^{2+} levels that accompanies traumatic SCI is one of the major mechanisms of secondary cell death; therefore, attenuation of post-traumatic accumulation of Ca^{2+} might afford some neuroprotection. The level of intracellular Ca^{2+} depends on the balance between systems for Ca^{2+} influx and efflux. Aspects of the influx and efflux pathways are shown to be affected by spinal cord contusion injury (e.g., L-type Ca^{2+} channels and the Ca^{2+}-ATPase pump).

MCP-1/Scya2 was increased both in the Epicenter sample and in a caudal–rostral wave beginning at 3 h and extending through 24 h post-SCI. Likewise, macrophage inflammatory protein MIP-1β/Scya3, which acts on lymphocytes (Schall et al., 1993), was increased at the Epicenter 3 and 24 h post-injury, and MIP-1α/Scya4 was increased at 3 h in the Epicenter (see Figs. 4 and 5). Cell adhesion molecules play important roles in the sequestering of immune cells to the walls of the vasculature at the sites of inflammation to allow cell extravasation from tissue (Haapasalmi et al., 1995). There are four main groups of these proteins: the integrins, the intercellular adhesion molecules (ICAMs) and vascular adhesion molecules (VCAMs), the selectins, and the cad-herins. P-selectin, ICAM-1 and VCAM-1 mRNAs were found to increase at 3 h after SCI, and ICAM-1 was also upregulated at 24 h.

Genes related to phagocytosis and induction of the complement cascade

Phagocytosis is initiated by the interaction of opsonins with specific receptors on the surface of macrophages and neutrophils. These receptors are from two families: the Fc receptors (FcR), which bind the Fc part of immunoglobulins

(Ravetch and Bolland, 2001), and the complement recep-tors, which bind complement on opsonized particles (Brown, 1992). The mRNAs for FcR receptors, FcγRII and FcγRIII, both low-affinity receptors, were increased within the injury epicenter 3–24 h post-SCI (see Fig. 4). A second increase in FcγRIII mRNA was observed 35 days after SCI. Induction of the classical complement pathway begins with the antibody-dependent binding of C1q to the Fc portion of IgG. C1qb mRNA was increased in the Rostral and Caudal samples by 24 h post-SCI and persisted through 35 days post-injury (see Fig. 4). A secondary increase in C1qb was observed in the Epicenter 35 days post-SCI. C3 mRNA was increased caudal to the lesion epicenter 7 days post-SCI and C4a was increased at the lesion epicenter 35 days post-SCI. C5 receptor 1 (C5r1) was increased in the Epicenter 7 days post-SCI.

Angiogenesis-related genes

Spinal cord trauma damages vascular cells, resulting in blood–spinal cord barrier permeability and the subsequent repair and formation of new blood vessels (angiogenesis). Few studies have examined vascular density changes after acute contusive/compressive SCI. However, it has been

Table 5
Summary table of fold changes in synaptic plasticity genes

Biological function	Gene name	Accession number	Fold change			
			3 h	24 h	7 days	35 days
NT biosynthesis	Tyrosine 3 monooxygenase/ tryptophan 5 monooxygenase activating protein	D17445	−1.1	−1.2	−1.1	−1.4[a]
NT biosynthesis	CaM kinase II gamma	J04063	−1.8	−1.9[a]	−2.8[a]	−1.7[a]
NT biosynthesis	Phenylethanolamine N-methyltransferase	U11275	1.3	1.0	1.6	−1.5[a]
NT biosynthesis	Glutamate decarboxylase (GAD 67)	X57573	−1.4	−1.2	−1.7	−2.3[a]
Vesicle protein	Synaptophysin	X06655	−1.2	−1.4	−1.3	−1.7[a]
Vesicle protein	Synapsin I	M27812	−1.6[a]	−1.5	−1.5	−1.9[a]
Vesicle protein	Synapsin II	M27925	−1.6[a]	−1.8[a]	−2.2[a]	−2.4[a]
Vesicle protein	Synaptic glycoprotein SC2	S45663	−1.1	−1.4	−1.5[a]	−1.6[a]
Vesicle protein	Synaptobrevin 2	M24104	−2.0[a]	−1.8[a]	−2.0	−2.1[a]
Vesicle protein	Syntaxin 2	M95735	−1.2	−1.5	−1.6	−1.8[a]
Vesicle protein	Synaptic vesicle glycoprotein 2a	L05435	−1.3	−1.4	−1.6[a]	−1.7[a]
Vesicle protein	Synaptic vesicle glycoprotein 2b	L10362	−2.0[a]	−3.4[a]	−3.8[a]	−1.1
		AA858626	−2.0	−1.1	−1.0	3.4[a]
Vesicle protein	Synaptotagmin 4	U14398	−1.6[a]	1.6[a]	−1.2	−1.0
Vesicle docking	SNAP 25A	AB003991	−1.5	−1.4[a]	−1.2	−2.0a
NT reuptake	GABA transporter protein	AI228669	−1.1	−1.8[a]	−1.3	−1.5
NT degradation	Monoamine oxidase B	M23601	−1.2	−2.1[a]	−1.2	1.0
NT degradation	Acetylcholinesterase T subunit	S50879	−1.5	−1.7[a]	−1.5	−1.6[a]
Neuromodulation	Glycine transporter 1	L13600	−1.1	−2.1[a]	−1.4	−1.2
Cation channel	Voltage-gated Ca^{2+} channel	M86621	1.3	−1.1[a]	−2.2[a]	−3.2[a]
NT receptor	Ionotropic glutamate receptor, AMPA2 (alpha2)	M38061	−1.5	−2.1[a]	−1.6	−1.7
Ca^{2+} signaling	G protein GO, alpha subunit	S80376	−1.9	−2.0	−3.5[a]	−2.3[a]
Ca^{2+} related signaling	Phospholipase C (PLC) beta 1	L14323	−1.3	−1.7[a]	−1.6	−1.6
		M20636	−2.0[a]	−3.5	−2.4	−2.4
Ca^{2+} pump	Ca^{2+}-ATPase	J03754	−1.3	−2.2[a]	−2.1	−2.0[a]
Ca^{2+} pump	Ca^{2+}-ATPase	J05087	−1.5	−1.8[a]	1.6	−1.6

NT = Neurotransmitter.
All comparisons are between contusion and laminectomy controls in the Epicenter sample.
[a] Denotes significant change detected by at least one method.

reported that, at the injury site, vascular density is decreased over the course of hours to a few days, followed by an increase in density, angiogenesis, around 4–7 days. (Casella et al., 2002; Loy et al., 2002; Vaquero et al., 1999; Zhang and Guth, 1997). Fig. 5 depicts the relationships between angiogenesis-associated genes that changed early after SCI, including TGF-β1 and angiopoietin, both of which increased in the Epicenter sample, and pleiotrophin, which decreased. Hypoxia-induced gene (HIG) showed decreases in all three spinal cord areas sampled, but only at 35 days, possibly indicating a return of adequate tissue perfusion. The gap in the time points (between 7 and 35 days) evaluated may not have allowed a clear picture of this evolving process.

Genes coding for proteins that influence apoptosis

There were a substantial number of changes in genes associated with the regulation of apoptosis (summarized in Fig. 6). We observed that SCI altered the transcription levels of two downstream effector caspases (3 and 7). Caspase 3 was upregulated in the Epicenter at 24 h, which is consistent with the results of Citron et al. (2000). Caspase 7 was downregulated at 3 h in the Epicenter and Caudal samples and then upregulated at 7 and 35 days in all three regions. Caspase 7 protein plays a central role in regulation of apoptosis. The decrease of caspase 7 observed at 3 h could be due to the simultaneous upregulation of the anti-apoptotic heat shock protein 70 (HSP70) and of nuclear factor kappa B (NF-κB), which inhibits caspases 3 and 7. Upregulation of HSP70 and NF-κB has been shown by others after similar SCI studies (Bethea et al., 1998; Carmel et al., 2001; Mautes and Noble, 2000). Caspases 3 and 7 induce DNA fragmentation, inhibit DNA repair, and cause cell shrinkage and membrane blebbing via Rock1 (upregulated at 35 days, Epicenter and Caudal samples); all of these events are a part of apoptosis. DNA damage, via p53, causes the transcriptional activation of Bax (upregulated at 35 days, Epicenter). Bax transcripts have been shown to be upregulated after SCI (Carmel et al., 2001). Both pro-apoptotic factors Bax and Bak-1 (upregulated at 7 days, Epicenter)
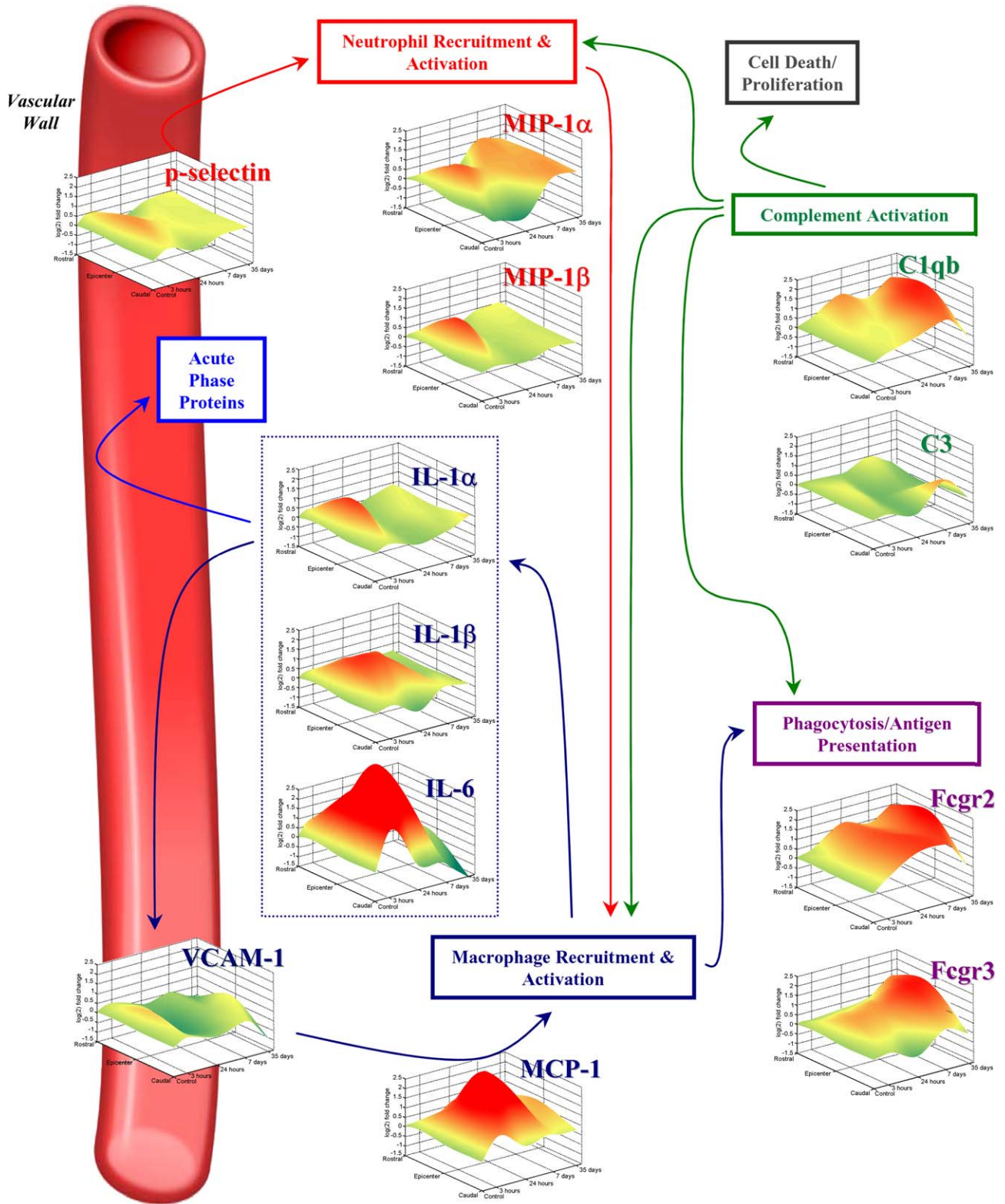
Fig. 4. Interaction of immune-related processes such as complement activation, phagocytosis/antigen presentation, macrophage recruitment, and neutrophil recruitment. Three-dimensional graphs represent the expression profiles of genes related to immune system activation following SCI.
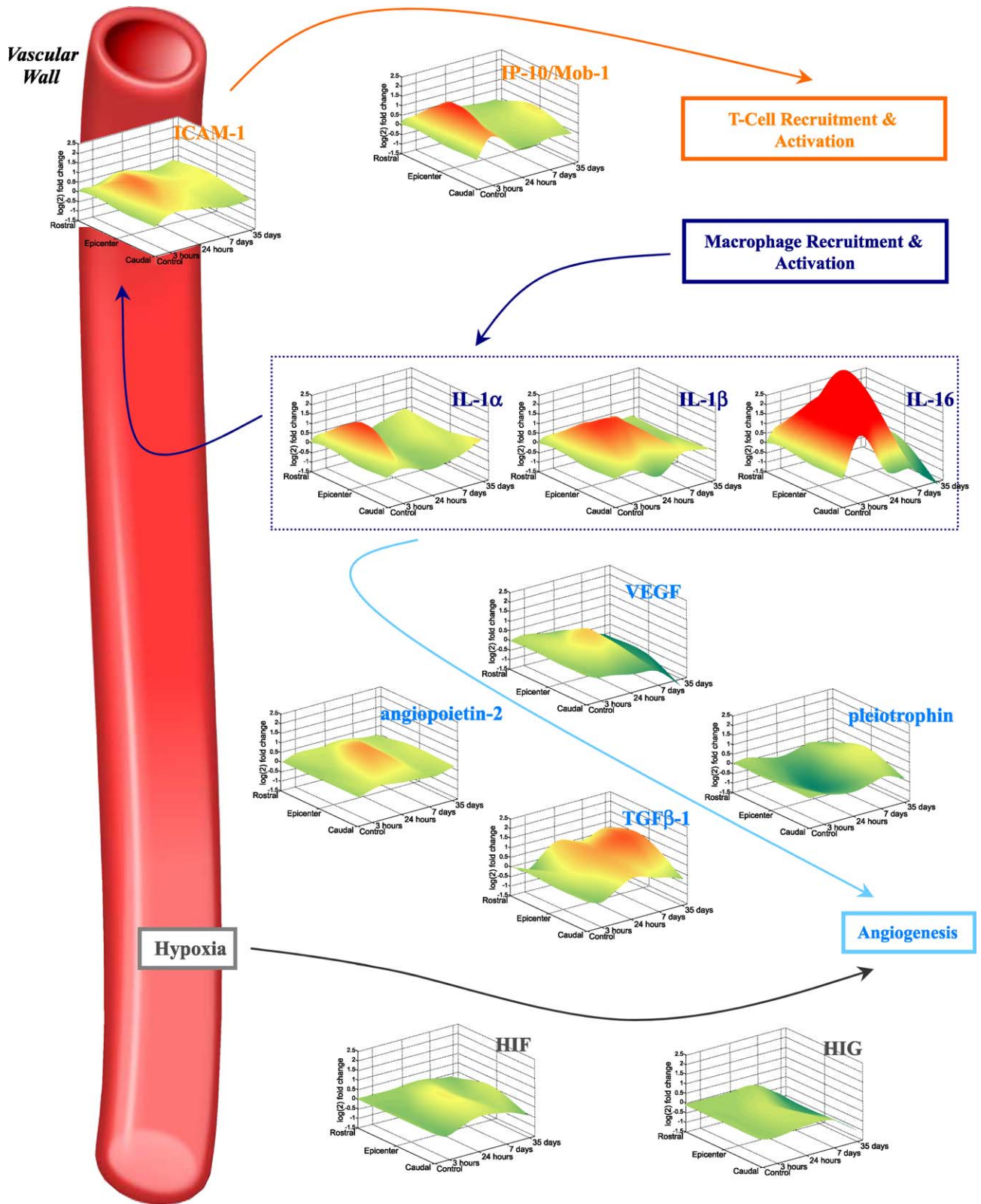
Fig. 5. Spinal cord trauma damages vasculature, causing subsequent angiogenesis. Several angiogenesis-associated genes changed early after SCI, including TGF-β, angiopoietin, pleiotrophin, and hypoxia-induced gene (HIG).
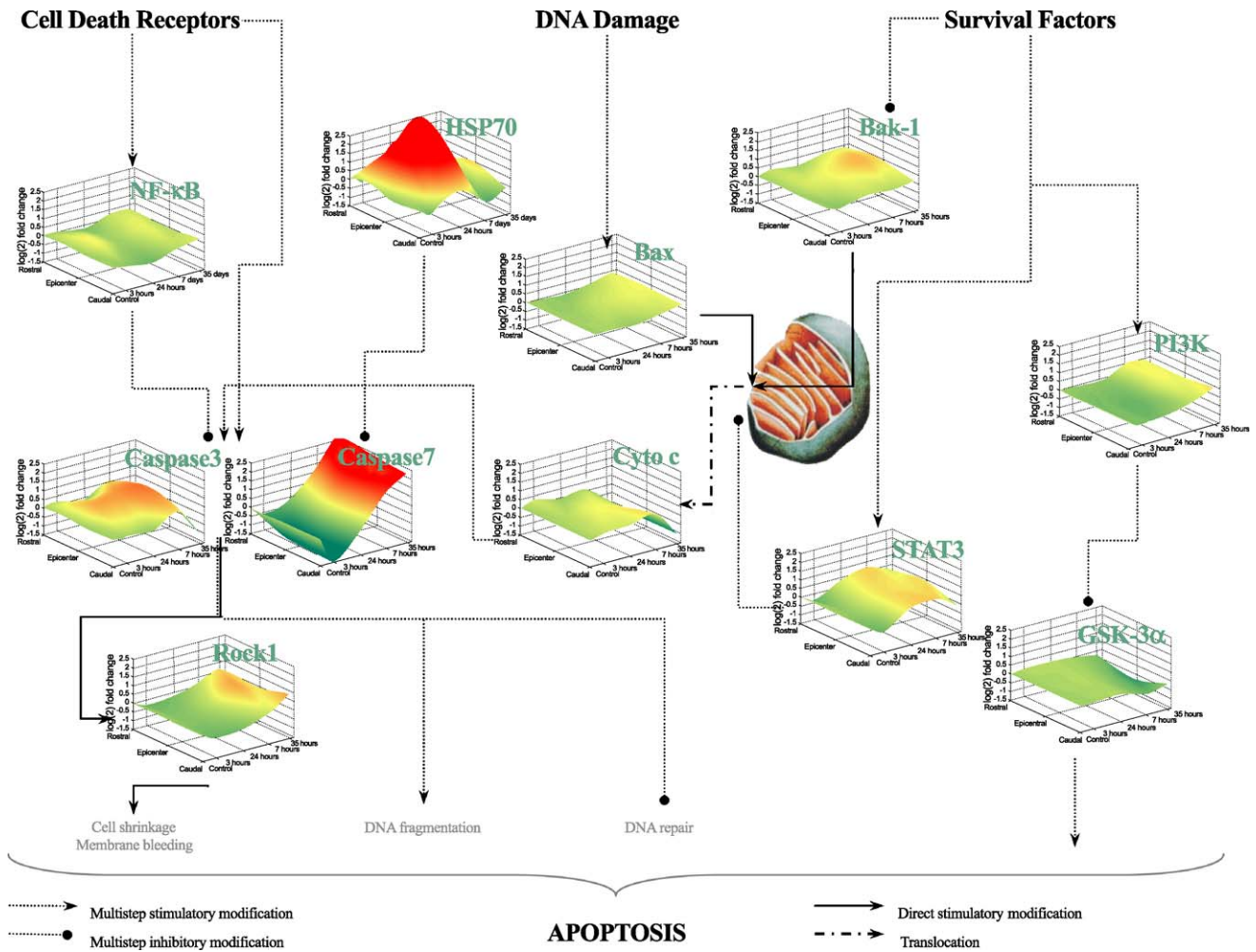
Fig. 6. Expression patterns and interrelationships of genes related to apoptosis. Phosphoinositide 3 kinase (PI3K), which is upregulated in the Epicenter sample 35 days after injury, is part of a pathway that inhibits pro-apoptotic Bcl-2 family member Bad, and inhibits additional apoptotic pathways linked to glycogene synthetase kinase-3 (GSK-3α, downregulated at 35 days). Survival factors such cytokines or growth factors activate Stat3 (upregulated caudal to the injury at 7 days), which prevents cytochrome *c* release and subsequent caspase activation, via induction of Bcl-2 and Bcl-xl.

trigger the release of cytochrome *c* (downregulated at 35 days, Rostral) from the mitochondria. Cytochrome *c* release leads to activation of caspases 3 and 7. The balance of pro- and anti-apoptotic events will determine whether a cell survives or dies. Cell survival requires the active inhibition of apoptosis, which is accomplished either by inhibiting caspases or by preventing their activation (for review, Budihardjo et al., 1999). The phosphoinositide 3 kinase (PI3K) pathway is activated by many survival factors (e.g., growth factors, cytokines), leads to the inhibition of pro-apoptotic Bcl-2 family member Bad, and inhibits additional apoptotic pathways linked to glycogen synthase kinase-3 (GSK-3). This study showed PI3K upregulation and GSK-3α downregulation at 35 days, consistent with the activation of a survival program. Additionally, survival factors cause the activation of the signal transducer and activator of transcription 3 (Stat3, upregulated at 7 days, Caudal) that prevents cytochrome *c* release and subsequent caspase activation, via induction of Bcl-2 and Bcl-xl.

Oxidative stress-related genes

Our data revealed increased expression of genes associated with antioxidant actions (see Fig. 7). The genes for the reactive oxygen species (ROS) scavengers metallothionein I and II (MT I and II) and genes associated with pathways that induce MT I and II were robustly upregulated in the Epicenter sample at 3 h, 7 days and 35 days after SCI (see also Table 4). Expression of genes coding for upstream regulators of the metallothioneins, including the cytokines IL-6, IL-1β, and TGF-β, was also increased. Expression of the gene for IL-6, which contributes to the regulation of inflammation as well as to the induction of MT I and II, was increased in the epicenter at 3 and 24 h, preceding the most robust increase of MT I and II. In addition, mRNA of genes coding for TGF-β and IL-1β is also more abundant at 24 h. TGF-β can induce IL-6 (Junn et al., 2000), which can in turn induce MT I and II. In contrast to the rapid upregulation of MT I and II and the transcripts of their upstream
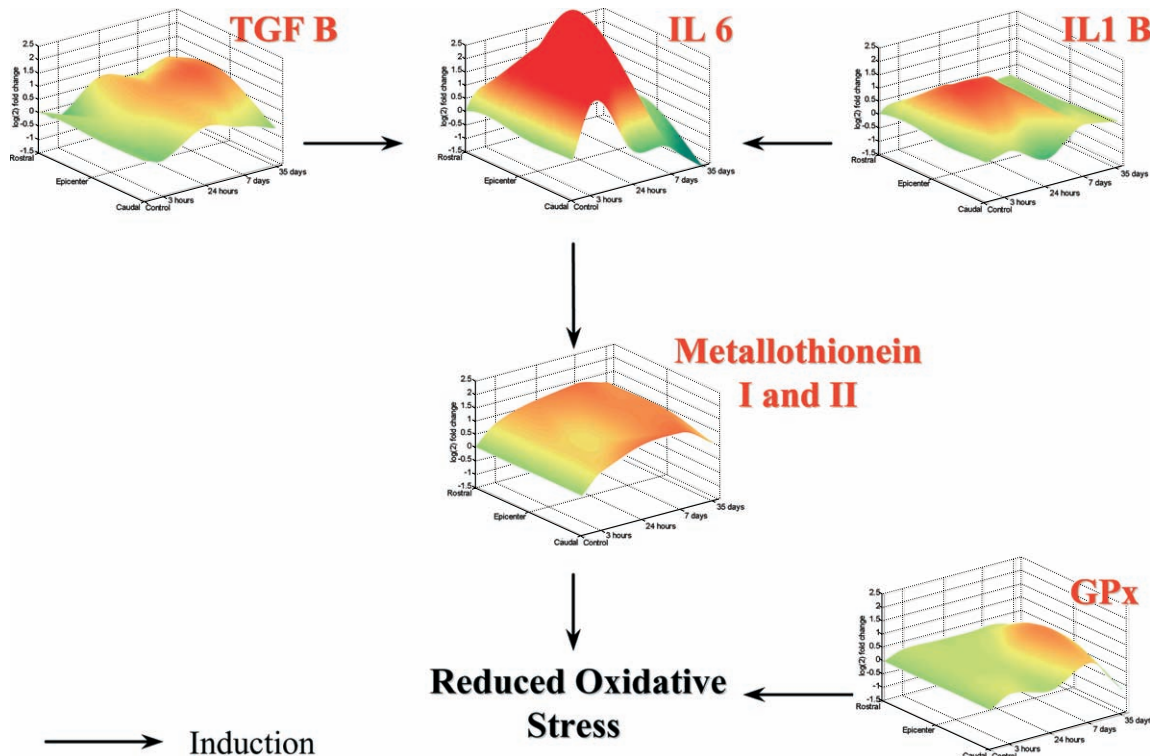
Fig. 7. In the Epicenter sample, reactive oxygen scavengers MT I and II remain upregulated 35 days after injury, as do their upstream regulators, IL-6, IL-1β, and TGF-β. The gene coding for glutathione peroxidase, another antioxidant, also remains chronically upregulated after injury.

regulators, there was a delayed increase rostrally and caudally in expression of genes for superoxide dismutase 1 (SOD1), SOD2, catalase and glutathione peroxidase (GPX), all of which have been shown to limit oxidative damage when administered after SCI (Santoscoy et al., 2002; Sugawara et al., 2002).

Long-term increased expression of genes coding for cathepsin proteases

We report here, for the first time, that after contusive SCI cathepsin L was upregulated in the Epicenter both at 24 h and 35 days. In contrast, cathepsins B, C, D, K, and S were upregulated only in the Epicenter at 35 days. Upregulation of cathepsins D and L was verified using Q-PCR, whereas the Q-PCR results did not confirm upregulation of cathepsin B. Endogenous cathepsin inhibitors in the Epicenter sample were variously upregulated (nexin at 3 and 24 h, cystatin S at 35 days) or downregulated (cystatin C, 24 h).

Discussion

Comparison with previous studies

The pathological changes following contusive SCI have been thoroughly documented. Axonal loss, readily apparent within 48 h after injury (McTigue et al., 1998), creates a fluid-filled cyst that persists in the chronic state, with a slim rim of spared tissue surrounding the cavity. Cavitation is also present rostral and caudal to the injury epicenter (Stokes and Reier, 1992). Neuronal and glial cell loss is apparent in the lesion epicenter, and rostral and caudal to it within 4 h of injury (Grossman et al., 2001). Damage to the cord also results in extensive proliferation of cells in and around the epicenter, many of which are microglia and macrophages (McTigue et al., 2001).

Our microarray data reflected these results and pinpointed many SCI-induced gene changes that have been described previously. For example, our data showed a similar upregulation of immediate early and cell cycle genes (e.g., cyclin D1, gadd45, krox24, NGFI-B) as well as genes related to oxidative stress and inflammation as described by Di Giovanni et al. (2003). Furthermore, osteopontin, a gene that was first described by Di Giovanni et al. (2003) to be upregulated at 4 and 24 h after SCI was significantly upregulated at 24 h and 35 days in the Epicenter and Caudal segments in our experiment. Our data also uphold results obtained by many prior studies of the immune response to SCI, including the increased expression of IL-1α and β, MCP-1, MIP-1α and β, p-selectin and ICAM-1 (Bartholdi and Schwab, 1997; Hamada et al., 1996; Hostettler and Carlson, 2002; Lee et al., 2000; Ma et al., 2002; Pan et al., 2002; Streit et al., 1998; Taoka et al., 1997). Interleukin-1 (IL-1) is a major mediator of inflammation and cell death pathways in the CNS after brain injury and ischemia (Boutin

et al., 2003; Hostettler and Carlson, 2002) and mediates induction in the spinal cord of COX-2, a mediator of arachidonic acid metabolism and prostaglandin synthesis (Tonai et al., 2002). RT-PCR and the RNase protection assay have shown that IL-1α and IL-1β mRNA increase after rat SCI (Hostettler and Carlson, 2002; Pan et al., 2002; Streit et al., 1998) and an increase in IL-1β protein has been shown by ELISA after contusive SCI (Nesic et al., 2001). Previous studies have shown increased MCP-1 and MIP-1α mRNA expression within hours of contusion-induced SCI in rats (Lee et al., 2000; Ma et al., 2002). Similar results have been reported for MIP-1α, MIP-1β, and IL-10 by in situ hybridization (Bartholdi and Schwab, 1997). MIP-1α and MCP-1 mRNA expression was upregulated in association with lysophosphatidyl choline-induced demyelination in the spinal cord, and neutralizing antibodies directed against these proteins or chemokine receptor antagonists decreased T-cell, neutrophil, and monocyte recruitment and attenuated myelin loss (Ghirnikar et al., 2000; Ousman and David, 2000).

We also observed increased expression of genes coding for cell adhesion molecules such as p-selectin, ICAM-1, and VCAM-1. Studies by Hamada et al. (1996) have shown that ICAM-1 mRNA expression correlates with the severity of injury to the spinal cord. They also demonstrated that the expression of ICAM-1 peaked at 6 h and that the acute introduction of antibodies against ICAM-1 could significantly improve recovery after SCI. P-selectin has also been shown to be expressed acutely after SCI, and antibodies directed against p-selectin can reduce neutrophil infiltration and histopathological changes after compressive SCI (Taoka et al., 1997).

Apoptosis-related and oxidative stress-related gene changes observed in our study also corroborate earlier findings, for example, the upregulation of HSP70, caspase 3, NF-κB, and metallothioneins (Bethea et al., 1998; Carmel et al., 2001; Citron et al., 2000; Mautes and Noble, 2000). TGF-β mRNA has been shown to increase at 24 h after SCI and to remain elevated at 7 days, and exogenous administration of TGF-β 30 min after SCI can decrease lesion size (Tyor et al., 2002). TNF-α can induce IL-6, and our data showed that TNF-α mRNA was increased at 3 h, but not at 24 h or 7 days post-injury. Increases in expression of genes coding for IL-6 and IL-1β have been reported at 2 and 24 h following contusive SCI (Pan et al., 2002). Similar changes in MT I and II, IL-6, and IL-1β were also reported in a previous experiment that examined gene expression following SCI (Carmel et al., 2001). Our analysis also pinpointed a previously unreported change in caspase 7 expression, which we found to be downregulated in the Epicenter and Caudal samples, which was corroborated by Q-PCR.

Our analysis also showed changes in genes that are related to neurotransmitter release, transport, and uptake, some of which are changes that have been previously reported. For example, the GABA transporter is involved in the uptake of GABA at the synapse following neuro-

transmitter release (Snow et al., 1992), and its mRNA was decreased in our Epicenter sample. This finding is consistent with the work of Tachibana et al. (2002) and Song et al. (2001) who examined the GABA transporter gene 24 h after injury. We detected altered expression of genes associated with formation and docking of presynaptic vesicles, such as SNAP 25A and synapsin II, both of which have previously been reported to be altered in a model of contusive SCI (Carmel et al., 2001; Johnson and Ascher, 1987). The fact that expression levels of genes associated with presynaptic vesicles were changed as late as 35 days after injury suggests that this is a chronic state (see Table 5).

There were several genes that have previously been shown to be differentially expressed after SCI that were not considered significant changes in our experiment, for example, GAP-43. One of the complications of the GeneChip system is its suboptimal ability to detect low-abundance transcripts in complex tissues. This is especially true of transcripts only expressed in small groups of cells. The size of the tissue segments used in this experiment (1 cm long) and the heterogeneity of the spinal cord make it highly likely that our study failed to reveal some of the changes that occur in certain subpopulations of cells. Finally, while our analytical approach was designed to minimize false negatives, it is inevitable that there were some real expression changes that were below our detection thresholds due to the inherent biological noise of an injury model.

Validation of analytic methods

Several of the genes described in this work have not yet been implicated in SCI, suggesting that additional validation was needed to confirm the changes seen. We validated a subset of the probe sets using Q-PCR to support the conclusions we have drawn. These results demonstrated the advantages of a multiple-method analysis approach. For example, three of the gene expression changes validated by Q-PCR were only found by one analysis method, while only four genes confirmed by Q-PCR were detected by all four algorithms. If the analysis had been limited in scope to just one method with strict criteria, several of the interesting results described here may not have been gleaned from our data. The decision to use methods with different underlying assumptions had its predicted results, with each method other than MAS 4.0 returning a substantial number of genes missed by the other approaches. For example, the Drop Method detected several subtle gene changes that were compelling but slightly below the 1.2-fold change cutoff used in other methods. Of the four analysis methods we used, dChip detected the most gene expression changes, possibly because its model approach may extract more signal from probe noise than the other approaches. Therefore, the multiple-method approach facilitated this experiment's ability to reveal novel areas of interest in SCI.

We included tissue samples distal to the lesion in our experimental design (Rostral and Caudal samples). This

proved to be a powerful technique because it allowed us to determine that some injury-induced perturbations are widespread in the spinal cord. For example, changes in the expression of genes coding for synapsin I, synapsin II, synaptobrevin 2, synaptic vesicle glycoprotein 2a, and synaptic vesicle glycoprotein 2b decreased in our Rostral and Caudal samples (see Table 5 for details), suggesting that the effect of SCI on synaptic proteins is not limited to the injury site. In addition, the changes seen in cholesterol metabolism and several apoptosis genes suggest these effects are not localized exclusively to the injury site, but to regions both rostral and caudal to the injury as well.

Limitations of experimental approach

We have explored how various gene changes might relate to one another within particular molecular pathways or processes; however, we accept that care needs to be taken when inferring causation from correlation. Our data result from comparisons made between large, multicellular chunks of nervous tissue; causal links between various gene changes cannot be established at the level of a particular cell (or indeed, at a particular pre-synaptic locus). Also, changes in gene expression do not always translate to modifications at the protein level, making it difficult to predict how a given gene change affects any particular pathway. Additional experiments will be required to determine whether one particular gene change has a causal impact on another gene or protein. Another important consideration is that some of the changes seen in the Epicenter sample, the area immediately surrounding the impact site, may be due to cell loss and/or invasion of new cells, although this is less of a concern in our Rostral and Caudal samples. Notwithstanding these caveats, we have attempted to illuminate potential links between, or therapeutic significance of, the various gene changes that we have observed.

Clinical ramifications

Despite the potential limitations of this microarray study, our spatiotemporal approach and powerful analysis tools have allowed us to identify several novel potential targets for therapeutic intervention after SCI. Our analysis revealed the persistent and extensive decrease in expression of HMG-CoA reductase, the gene coding for the rate-limiting enzyme for cholesterol synthesis. The biosynthesis of cholesterol within the central nervous system is a tightly regulated and complex process that requires dozens of enzymes. It has been estimated that myelin contains 70% of the cholesterol present within the brain (Snipes and Suter, 1997), but there is little discussion in the literature of the relationship between lowered cholesterol levels in the spinal tissue and dysmyelination after spinal injury, although changes in lipid metabolism, including the loss of cholesterol in the spinal cord (Demediuk et al., 1985), have been reported. Sponta-

neous partial remyelination of fibers has been described after contusive spinal injury (Salgado-Ceballos et al., 1998), but loss of myelin in remaining axons is nonetheless considerable (Blight, 1983), and represents a major barrier towards restoration of function. Furthermore, the myelin present on remaining axons is often provided by Schwann cells (Blight, 1983; Blight and Young, 1989) that have entered the spinal cord primarily from the spinal roots due to damage of the glial limiting membrane (Bunge et al., 1994; Takami et al., 2002). Under normal circumstances, the blood–spinal barrier (BSB) prevents uptake of cholesterol, which must therefore be synthesized de novo (Jurevics and Morell, 1995). The BSB breakdown for macromolecules lasts approximately 14 days after SCI (Noble and Wrathall, 1989; Schnell et al., 1999), thus, it is unlikely that cholesterol can enter the spinal tissue from sources outside the CNS after this time. We hypothesize, therefore, that reestablishing a normal level of local cholesterol synthesis, either by replacing myelin-producing cells or by increasing expression of cholesterol synthesis genes in these cells within the injured cord, may enhance axonal conduction and, perhaps, synapse formation or function, resulting ultimately in improved functional outcome.

Our analysis revealed that many genes associated with calcium signaling were disrupted, which may be particularly important considering the large body of evidence suggesting that increased intracellular calcium is one of the main causes of secondary cell death following SCI (Happel et al., 1981; Sribnick et al., 2003; Tymianski and Tator, 1996). The system that controls intracellular calcium consists of voltage-gated Ca^{2+} channels (e.g., L-type) and metabotropic Ca^{2+} channels (e.g., NMDA and AMPA), both of which lead to calcium influx, the Na^{2+}/Ca^{2+} exchanger that is active in both calcium influx and efflux, and finally the Ca^{2+} pump, Ca^{2+}-ATPase, which we found to decrease with injury. Among these systems, sodium/calcium exchanger and Ca^{2+}-ATPase function to release calcium from the cell (Mata and Fink, 1989; Regehr, 1997). Unlike the sodium/calcium exchanger that controls intracellular calcium based on the relative concentration gradients of sodium and calcium, Ca^{2+}-ATPase uses ATP to pump calcium against its electrochemical gradient. Although the function of Ca^{2+}-ATPase is well defined in the brain and our study revealed many genes for Ca^{2+}-ATPase isoforms within the spinal cord, Ca^{2+}-ATPase has not been examined as a potential therapeutic target following SCI. Our data also suggest a robust downregulation of several other Ca^{2+}-related genes, including voltage-gated (L-type) Ca^{2+} channels, Ca^{2+}-permeable ionotropic glutamate (AMPA) channels, and other Ca^{2+}-dependent intracellular signaling molecules such as G protein G0 (alpha subunit) and phospholipase C (see Table 5), suggesting that therapeutics designed to facilitate active calcium efflux could foster recovery. One potential mechanism by which this could be accomplished is through the activation of Ca^{2+}-ATPase at the injury site; however, current methods involve enzymes that lead to the activation

of calmodulin (e.g., methionine sulfoxide reductase), which is problematic due to calmodulin's implication in nitric oxide synthase activation (Dawson et al., 1995; Sun et al., 1999).

To our knowledge, this study described for the first time alterations of gene expression for certain members of the cathepsin protease family, including L, C, D, K, and S. Very little is known about spatiotemporal changes in the cathepsin family of intracellular enzymes following contusive SCI. This is remiss given that these enzymes have been linked with neuronal and glial death in various models of nervous system injury and disease (Uchiyama, 2001; Yamashima, 2000). Recently, however, it has been shown that levels of cathepsin B (mRNA, proenzyme, and active enzyme) increase after contusion injury to the adult rat spinal cord (Ellis et al., 2004). Post-SCI changes have also been described at the protein activity level for cathepsins—B-like, D and neutral (Banik et al., 1986; Yashon et al., 1975). No information exists for changes at the gene expression level for any other of these enzymes. Further work is in progress to reveal which cell types express cathepsin genes and which of the gene alterations are translated to changes at the protein level. Our data suggest that any pathological role for cathepsins at the injury site earlier than 35 days post-injury must predominantly result from the net activity of enzymes synthesized before the insult. We suggest that any attempt to overcome putative cathepsin-mediated cell death in the acute phase should be made via targeting of existing enzyme proteins, rather than by targeting the post-injury synthesis of these enzymes (since increased synthesis occurs well after the majority of cells are already lost). Existing cathepsin inhibitors, such as E-64c or CP-1, could be tested for efficacy in treating contusive SCI since they reduce cell death and neurological deficits when delivered intravenously following induction of ischemia in rodents and primates (Seyfried et al., 2001; Tsuchiya et al., 1999; Yoshida et al., 2002).

Our investigation of changes in expression of immune system-related genes suggested that enhancing the activity of the anti-inflammatory IL-1 receptor might be beneficial after SCI. For wound healing to occur, it is important that the immune response be switched off to allow extracellular matrix synthesis and remodeling to proceed. We observed increased expression of IL-1RII within the injury epicenter at 24 h post-injury. The type II IL-1 receptor is a non-signaling receptor that binds IL-1 but does not transduce a signal; thus, it acts as a "decoy trap" for IL-1 (Mantovani et al., 1997). The expression of IL-1 receptors has not been investigated after SCI, but our data indicate it to be a potential target for treatment of SCI-induced inflammation. In addition, our data suggest that FcRs may be promising therapeutic targets. FcγRII and FcγRIII were both increased in the Epicenter sample acutely (3–24 h), and FcγRIII was again increased at 35 days. FcγRIII is expressed almost exclusively on neutrophils (Garcia-Garcia and Rosales, 2002), and although the initial expression of

this receptor coincides with the peak of their infiltration, its later expression at 35 days, when neutrophils are no longer present, points to its expression on other cell types such as macrophages or microglia. The use of strategies modulating phagocytic receptor expression or activity is an area yet to be explored in SCI.

Examination of gene expression at 35 days after SCI proved to be very informative. Our analysis of expression changes at this chronic stage of injury suggested that several treatments for SCI that have been attempted acutely after injury should be continued long-term. For example, our data suggest that the complement cascade may be active as late as 35 days after injury. The classical pathway of complement activation is generally associated with the antibody-dependent binding of C1q to the Fc portion of IgG; however, molecules, including myelin, can directly activate complement (Eggleton et al., 1998; Morgan, 1995) Autocatalytic activation of complement cascade leads to formation of a membrane attack or terminal complement complex (termed MAC, TCC, or C5b-9), which can lead to a range of cellular consequences, from Ca^{2+} influx and alterations in signaling pathways to cell death (Morgan, 1989, 1995). We observed that expression of genes associated with the complement cascade remained increased at 35 days after injury, including C1qb, C4a, and C5r1. These long-term changes in the complement cascade suggest that inflammation-related effects are still present in a more chronic state and need to be addressed therapeutically.

Close examination of apoptosis-related genes within the context of our spatiotemporal design revealed that therapeutic interventions targeting apoptotic pathways should be targeted not only at the epicenter, but rostral and caudal of the injury and should last at least 5 weeks. We show that pro- and anti-apoptotic genes are altered as late as 35 days following SCI and that these changes occur distant from the site of injury. Programmed cell death, or apoptosis, is an inter-related collection of pathways and mechanisms used to eliminate excess or unwanted cells. Histological evidence (TUNEL staining, chromatin condensation, and DNA laddering) has shown that apoptosis involving both neurons and glia contributes to spinal cord tissue damage after traumatic insults (Liu et al., 1997). This cell death mechanism directly involves both pro- and anti-apoptotic proteins. Activation or upregulation of the pro-apoptotic molecules will trigger degeneration, whereas activation or upregulation of anti-apoptotic molecules can prevent the initiation or progression of degeneration (for review, Liou et al., 2003). Apoptosis can be induced via signaling through a family of receptors known collectively as "death receptors" (i.e., Fas, Tumor necrosis factor receptors). Death receptor ligands initiate the activation of caspase cascades, a family of cysteine proteases, which are central regulators of apoptosis (for review, Budihardjo et al., 1999). Our data are the first to demonstrate that pro- and anti-apoptotic transcripts are regulated as early as 3 h and as late at 35 days post-injury and as far as 1 cm rostral and caudal of the injury, though it

is less clear from our data which specific cells are being affected. These findings are consistent with a report showing that impact-induced spinal cord cell apoptosis was not confined in space to the immediate impact site or in time to the immediate post-injury period (Liu et al., 1997). In this context, several groups have reported a burst of neuronal and glial apoptosis in gray and white matter at the lesion site within 24 h and apoptosis of oligodendrocytes in the distant white matter as much as 2 weeks later (Liu et al., 1997; Springer et al., 1999). Because apoptosis is typically a rapid process, lasting hours, this late apoptosis likely reflects a wave of delayed oligodendrocyte death. The observation of gene changes consistent with apoptotic program 5 weeks post-injury has important therapeutic implications. In particular, oligodendrocyte loss during this late phase of cell death leads to axonal demyelination and dysfunction in areas distant from the injury epicenter, which contributes further to the long-term neurological deficits. To improve oligodendrocyte rescue, and significant recovery, our study suggests investigating the application of current treatments such as Bcl-2 gene transfer (Yukawa et al., 2002) or the caspase inhibitor z-VAD (Ozawa et al., 2002) at later time points (the latest time point of these studies was typically 72 h post-injury) and not only at the epicenter, but also rostral and caudal from the injury.

Oxidative stress, which results from accumulation of ROS, can trigger apoptotic events and is, therefore, a major contributor to secondary damage after SCI (for review, see Juurlink and Paterson, 1998; Waldmeier, 2003). ROS have been shown to be increased after SCI (Azbill et al., 1997). One source of this increase in free radical formation is the Ca^{2+} disruption of the mitochondrial electron transport chain. Disruption in mitochondrial function results in the release of pro-apoptotic signals, such as cytochrome *c* and apoptosis-inducing factor (AIF). After injury, these apoptotic events can be counteracted by increases in ROS scavengers or antioxidants and genes that induce these molecules. The genes for ROS scavengers metallothionein I and II (MT I and II) and genes associated with pathways that induce MT I and II, including IL-6, IL-1β, and TGF-β, were robustly upregulated in the Epicenter sample both early and late after SCI (see also Table 4). One mechanism of action for IL-6, believed to be a key regulator of cell survival after injury, may be through MT I and II. After a cortical freeze lesion in *IL-6−/−* mice, the inflammatory response was impaired and there was an increase in oxidative stress (Penkowa et al., 2000). These changes were coincident with a decrease in MT I and II protein but no changes in SOD, suggesting that MT I and II levels may be critical to counteracting oxidative damage after injury. Furthermore, exogenous addition of MT I and II in a model of experimental autoimmune encephalomyelitis reduces demyelination and stimulates oligodendrocyte regeneration (Penkowa and Hidalgo, 2003). The delayed increase in the antioxidants, catalase, SOD, and GPX reported here is supported by previous work (Azbill et al.,

1997). Our data thus suggest that the activation of MT I and II may be the initial endogenous attempt at combating oxidative stress after injury; therefore, targeting a free radical scavenger, such as MT I or II, in addition to anti-lipid peroxidation drugs, such as methylprednisolone, could be beneficial to limiting secondary injury.

In summary, many microarray studies are undertaken with the intent to discover new avenues for future study. Frequently, the focus of these studies are single gene targets, often involved in plasticity or neuroprotection in the case of SCI. Rather than focusing on the single gene level, we have endeavored to target functional classes of genes that code for proteins involved in processes integral to the evolution of SCI. By inspecting functional groups with an appropriately tailored analysis, we were able to suggest several novel potential therapeutic targets. Furthermore, our data allowed us to conclude that it is possible that several interventions, for example, anti-apoptotic treatments, should be maintained in the chronic phase of injury and in areas distal to the lesion epicenter.

Acknowledgments

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